



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US92/01383  <b>(22) International Filing Date:</b> 21 February 1992 (21.02.92)  <b>(30) Priority data:</b> <table border="0"> <tr><td>658,796</td><td>21 February 1991 (21.02.91)</td><td>US</td></tr> <tr><td>658,849</td><td>21 February 1991 (21.02.91)</td><td>US</td></tr> <tr><td>659,103</td><td>21 February 1991 (21.02.91)</td><td>US</td></tr> <tr><td>659,113</td><td>21 February 1991 (21.02.91)</td><td>US</td></tr> <tr><td>659,114</td><td>21 February 1991 (21.02.91)</td><td>US</td></tr> <tr><td>659,980</td><td>21 February 1991 (21.02.91)</td><td>US</td></tr> <tr><td>659,981</td><td>21 February 1991 (21.02.91)</td><td>US</td></tr> <tr><td>744,870</td><td>14 August 1991 (14.08.91)</td><td>US</td></tr> <tr><td>745,215</td><td>14 August 1991 (14.08.91)</td><td>US</td></tr> <tr><td>787,921</td><td>6 November 1991 (06.11.91)</td><td>US</td></tr> </table> <b>(71) Applicant (for all designated States except US):</b> GILEAD SCIENCES, INC. [US/US]; 344 Lakeside Drive, Foster City, CA 94404 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> TOOLE, John, J. [US/US]; 1617 Forest View Avenue, Burlingame, CA 94010 (US). GRIFFIN, Linda, C. [US/US]; 56 Walnut Avenue, Atherton, CA 94027 (US). BOCK, Louis, C. [US/US]; 342 Stanchion Lane, Foster City, CA 94404 (US). LATHAM, John, A. [US/US]; 1160 Byron Street, Palo Alto, CA 94301 (US). MUENCHAU, Daryl, Dean [US/US]; 98 Rockharbor Lane, Foster City, CA 94404 (US). KRAWCZYK, Steven [US/US]; 3149 Casa De Campo, San Mateo, CA 94403 (US).		658,796	21 February 1991 (21.02.91)	US	658,849	21 February 1991 (21.02.91)	US	659,103	21 February 1991 (21.02.91)	US	659,113	21 February 1991 (21.02.91)	US	659,114	21 February 1991 (21.02.91)	US	659,980	21 February 1991 (21.02.91)	US	659,981	21 February 1991 (21.02.91)	US	744,870	14 August 1991 (14.08.91)	US	745,215	14 August 1991 (14.08.91)	US	787,921	6 November 1991 (06.11.91)	US	<b>(74) Agents:</b> MURASHIGE, Kate, H. et al.; Morrison & Foerster, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).  <b>(81) Designated States:</b> AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC (European patent), MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, RU, SD, SE, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent), US.  <b>Published</b> <i>With international search report.</i>
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<b>(54) Title:</b> APTAMERS SPECIFIC FOR BIOMOLECULES AND METHOD OF MAKING  <b>(57) Abstract</b>  <p>A method for identifying oligomer sequences which specifically bind target molecules such as serum proteins, kinins, eicosanoids and extracellular proteins is described. The method is used to generate aptamers that bind to serum Factor X, thrombin, bradykinin, PGF<sub>2</sub><math>\alpha</math> and cell surface molecules. The technique involves complexation of the target molecule with a mixture of oligonucleotides containing random sequences and sequences which serve as primer for PCR under conditions wherein a complex is formed with the specifically binding sequences, but not with the other members of the oligonucleotide mixture. The complex is then separated from uncomplexed oligonucleotides and the complexed members of the oligonucleotide mixture are recovered from the separated complex using the polymerase chain reaction. The recovered oligonucleotides may be sequenced, and successive rounds of selection using complexation, separation, amplification and recovery can be employed. The oligonucleotides can be used for therapeutic and diagnostic purposes.</p>																																

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5                    APTAMER SPECIFIC FOR BIOMOLECULES  
                    AND METHOD OF MAKING

Technical Field

                    The present invention is directed to a method  
10    for identifying oligonucleotide sequences which  
                    specifically bind biomolecules, including peptides,  
                    hydrophobic molecules, and target features on cell  
                    surfaces, in particular extracellular proteins, and the  
                    use of these sequences to detect and/or isolate the  
15    target molecules and the resulting compositions. The  
                    instant invention is exemplified by obtaining  
                    compositions, through the use of disclosed methods, that  
                    comprise oligonucleotide sequences which bind to Factor  
                    X, thrombin, kinins, eicosanoids and extracellular  
20    proteins.

                    The invention is also directed to improvements  
                    in methods to identify specific binding sequences for  
                    target substances and methods of use of such specific  
                    binding sequences. More specifically, it concerns: (1)  
25    the use of oligonucleotides containing modified monomer  
                    residues to expand the repertoire of candidate oligomer  
                    sequences; (2) the use of identifying and amplifying  
                    oligonucleotides without attached flanking regions or  
                    structural constraints, but which nevertheless are  
30    capable of specific binding to desired targets; and (3)  
                    the design and use of conjugates designed to bind  
                    specific target cells and induce an immune response to  
                    the target cells.

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Background and Related Art

Specifically Binding Oligonucleotides. Conventional methods of therapeutic treatment based on binding and inhibition of therapeutic target molecules as well as detection and isolation of proteins and other molecules have employed small molecules, antibodies and the like which specifically bind such substances. Recently, however, the de novo design of specifically binding oligonucleotides for non-oligonucleotide targets has been described. See, e.g., Blackwell et al., Science (1990) 250:1104-1110; Blackwell et al., Science (1990) 250:1149-1151; Tuerk, C., and Gold, L., Science (1990) 249:505-510; Ellington et al., Nature (1990) 346:818-822. Such oligonucleotides have been termed "aptamers" herein. The Tuerk reference describes the use of an in vitro selection and enrichment procedure to obtain RNA molecules that bind to an RNA binding protein. In this method, a pool of RNAs that are completely randomized at specific positions is subjected to selection for binding to a desired protein. The selected RNAs are then amplified as double-stranded DNA that is competent for subsequent in vitro transcription. The newly transcribed RNA is then enriched for better binding sequences and recycled through this procedure. The amplified selected sequences are subjected to sequence determination using dideoxy sequencing. Tuerk and Gold applied this procedure to determination of RNA molecules which are bound by T4 DNA polymerase. The method utilizes the polymerase chain reaction (PCR) technique, as described by Saiki, R.K., et al., Science (1988) 239:487-491, to amplify the selected RNAs.

Kinzler, K.W., et al., Nucleic Acids Res (1989) 17:3645-3653, describes the use of PCR to identify DNA sequences that are bound by proteins that regulate gene expression. In the reported work, total genomic DNA is

first converted to a form that is suitable for amplification by PCR and the DNA sequences of interest are selected by binding to the target regulatory protein. The recovered bound sequences are then amplified by PCR.

5 The selection and amplification process are repeated as needed. The process as described was applied to identify DNA sequences which bind to the Xenopus laevis trans-

10 cription factor 3A. The same authors (Kinzler et al.) in a later paper, Mol Cell Biol (1990) 10:634-642, applied this technique to identify the portion of the human genome which is bound by the GLI gene product produced as a recombinant fusion protein. The GLI gene is amplified in a subset of human tumors.

Ellington, A.D., et al., Nature (1990) 346:818-822, describe the production of a large number of random sequence RNA molecules and identification of those which bind specifically to selected molecules, for instance, organic dyes such as Cibacron blue. Randomly synthesized DNA yielding approximately  $10^{15}$  individual sequences was

20 amplified by PCR and transcribed into RNA. It was thought that the complexity of the pool was reduced in the amplification/transcription steps to approximately  $10^{13}$  different sequences. The pool was then applied to an affinity column containing the dye and the bound

25 sequences subsequently eluted, treated with reverse transcriptase and amplified by PCR. The results showed that about one in  $10^{10}$  random sequence RNA molecules folds in such a way as to bind specifically to the ligand.

30 Thiesen, H.-J., and Bach, C., Nucleic Acids Res (1990) 18:3203-3208, describe what they call a target detection assay (TDA) to determine DNA binding sites for putative DNA binding proteins. In their approach, a purified functionally active DNA binding protein and a

35 pool of genomic double-stranded oligonucleotides which

contain PCR primer sites at each end were incubated with the protein. The resulting DNA complexes with the protein (in their case, the SP1 regulatory protein) were separated from the unbound oligomers in the mixture by  
5 band-shift electrophoresis and the complex oligonucleotides were rescued by PCR and cloned, and then sequenced using double-stranded mini-prep DNA sequencing.

None of the above references, however, describes the identification of oligonucleotides which  
10 specifically bind biomolecules that are not known to interact with oligonucleotides. In particular, these references do not describe the identification of oligonucleotides which specifically bind peptide molecules such as serum proteins, kinins, hydrophobic  
15 molecules such as eicosanoids, or extracellular proteins.

In addition, the art has not demonstrated (i) in vivo therapeutic (mammalian or primate) efficacy of selected oligonucleotides for any clinical indication, (ii) binding of single-stranded DNA oligonucleotides to  
20 molecules that do not ordinarily bind to nucleic acid as part of their normal function, (iii) interference with the function of a target molecule by bound a oligonucleotide or aptamer, (iv) target molecule binding mediated by single-stranded DNA and (v) target-specific  
25 binding of short oligonucleotides or oligonucleotide analogs that are derived from a larger full-length parent oligonucleotide (aptamer) molecule.

Targets. Kinins are peptides which are formed in biological fluids by the activation of kininogens.  
30 Kinins have been shown to exert numerous physiological and pathological actions such as exhibiting hypotensive effects, causing pain, mediating reactive hyperaemia in exocrine glands, playing a role in vascular and cellular events that accompany the inflammatory processes,  
35 controlling blood pressure, and possibly acting as

protective agents against hypertension. In pathological states, kinins have been implicated in asthma, inflammatory diseases such as rheumatoid arthritis and other forms of arthritis, vascular changes occurring in migraine, myocardial infarction, cardiovascular failure, carcinoid and postgastrectomy dumping syndromes, hyperbradykininism syndrome, hemorrhagic and endotoxic shock, as well as other pathological conditions. For a review of kinins, see Regoli, D., and Barabe, J., Pharmacological Review (1980) 32:1-46.

Eicosanoids are a family of fatty acid derivatives which include the various prostaglandins, thromboxanes, leukotrienes and prostacyclin. Eicosanoids are widespread and produce a remarkably broad spectrum of effects embracing nearly every biological function. For example, eicosanoids have been shown to affect the cardiovascular system, blood, smooth muscle, kidney and urine formation, the central nervous system, inflammatory and immune responses, afferent nerves and pain, as well as several metabolic functions. For a general review of eicosanoids and their biological significance, see Moncada, S., et al., in The Pharmacological Basis of Therapeutics, Gilman, A.G., et al., eds. (MacMillan Publishing Company, New York), 7th Edition, pages 660-671.

Many of these molecules are so ubiquitous that antibody production in laboratory animals against the native molecules is difficult unless they are chemically modified to become antigenic. Labeled kinins with sufficient specific activity are not available and bradykinin antibodies tend to cross-react with kininogen. Therefore, conventional immunodiagnostic and isolation techniques are not easily available with respect to these substances. It would therefore be desirable to develop alternative methods for working with these agents.

Additionally, there are numerous difficulties related to collecting biological samples while avoiding the formation or the inactivation of kinins. Thus, previous assay methods have focused on measuring the particular kininogens rather than the activation peptides thereof. For a review of the problems associated with the use of conventional diagnostic techniques and kinins, see Goodfriend, T.L., and Ody, C.E., in Methods of Hormone Radioimmunoassays, B.N. Jaffee and H.R. Behrman, eds. (Academic Press, New York), 1979, pages 909-923; and Talamo, R.C., and Goodfriend, T.L., Handbook Exp. Pharmacol. (1979) 25 (Suppl.):301-309. It would therefore be desirable to develop alternative methods for working with these agents.

Particular cells can be characterized by the presence of certain proteins on their surface. These proteins can serve a variety of functions including providing binding sites for other biomolecules and/or virus receptors. It is also known that it is possible to differentiate normal cells of a given type from abnormal cells by the type and/or amount of characteristic protein on the cells' surface. Since it is known that it is possible to differentiate different types of cells by the characteristic proteins present on their surface, different methodologies have been developed in attempts to characterize cells by the ability of certain molecules to bind to the characteristic proteins on those cells.

The present inventors postulated that oligonucleotides could be used to bind to characteristic proteins at the cell surface. Although such binding does occur, it is not highly specific, i.e., a given oligonucleotide may bind to cellular proteins on two very different types of cell lines. Further, even if a particular oligonucleotide is found to be specific to a particular characteristic protein, it is difficult to



isolate the desired oligonucleotide and produce it in sufficient amounts so as to allow it to be useful as a probe to identify particular cell lines having particular characteristic proteins thereon.

5           The invention herein provides an approach and utilizes a binding selection method combined with PCR or other amplification methods to develop aptamers that bind peptide molecules such as factor X, kinins, hydrophobic molecules such as eicosanoids, and extracellular  
10 proteins. In this method, selected and amplified aptamers that specifically bind to these targets are obtained starting from a pool of randomized oligonucleotides.

Synthetic Methods. The above references on  
15 specifically-binding oligonucleotides do not suggest that oligomers can be synthesized in the candidate mixture containing analogous forms of purines and pyrimidines, as well as modifications in the sugar moieties and the phosphodiester linkages. This inclusion is significant,  
20 since those oligomers containing modifications may have superior binding qualities which are attributable to the modifications per se and this inclusion thus expands the repertoire of candidates subjected to the initial screen. The present invention is related in part to an  
25 improvement in the above-described methods wherein oligomers containing modifications not found in native sequences can be included among the candidates for specific binding.

          Furthermore, although PCR has made possible the  
30 isolation and analysis of specific nucleic acid fragments from a wide variety of sources, application of PCR to isolate and analyze a particular nucleic acid region heretofore has required knowledge of the nucleic acid sequences either flanking or within the region of  
35 interest. The requirement of prior knowledge of the

flanking region is particularly troublesome when trying to identify aptamers. Flanking primer sequences impose limits on aptamer structural diversity: either the ability to bind is affected by the primers, thereby eliminating from consideration a class of binding agents, or occasionally, the primers actually participate in or facilitate binding by conferring structure. Flanking sequence thus may impose constraints which make aptamers so identified suboptimal for drug development. These problems with the processes of selection for truly optimal binding agents or aptamers have severely limited drug development.

Clearly, it would be advantageous to devise methods which permit the identification of optimal aptamers. Methods such as those described by Ellington, A.D. et al., Nature (1990) 346:818-822, estimate that 1 in  $10^{10}$  aptamers bind in that system. The novel methods herein described and claimed may revise this ratio downward to 1 in  $10^9$  or 1 in  $10^8$ . With regard to drug development, many scientists have failed to recognize the problem that flanking primer sequences represent with respect to selection for truly optimal binding agents.

Furthermore, none of the cited references describe the identification of aptamers capable of binding to proteins such as thrombin, nor is the use of single stranded DNA suggested as an appropriate material for generating aptamers. The use of DNA aptamers according to this invention has several advantages over RNA including increased nuclease stability and ease of amplification by PCR or other methods. RNA generally is converted to DNA prior to amplification using reverse transcriptase, a process that is not equally efficient with all sequences, resulting in loss of some aptamers from a selected pool.

Modified Bases in Polymerization Reactions. A

large number of modifications which behave in a known manner in polymerase reactions is known. Otvos, L., et al., Nucleic Acids Res (1987) 1763-1777, report the enzyme catalyzed incorporation of 5-(1-alkenyl)-2'-deoxyuridines into DNA. As reported in this paper, 5-vinyl-dUP behaved in the DNA polymerase I reaction catalyzed by the Klenow fragment in a manner similar to dTTP; (E)-5-(1-heptenyl) and (E)-5-(1-octenyl)-dUDPs were poor substrates; however, all of these residues are read as thymidine in the polymerization.

Allen, D.J., et al., Biochemistry (1989) 28:4601-4607, report the incorporation of 5-(propylamino) uridine into oligomers and its labeling, using the propylamine function, with mansyl chloride. This complex was used to study interaction with DNA polymerase I (Klenow fragment) and was shown to interact with the enzyme. This base residue is also recognized as thymidine.

Langer, P.R., et al., Proc Natl Acad Sci USA (1981) 78:6633-6637, described the synthesis of DNA and RNA using dUTP and UTP residues labeled with biotin through a linker at the C5 position. These labeled forms of dUTP and UTP were utilized by a number of DNA and RNA polymerases and are recognized by these enzymes when included in the oligomer template as thymidine or uridine.

Gebyehu, G., et al., Nucleic Acids Res (1987) 15:4513, reported biotin-labeling of dATP and dCTP nucleotide analogs through the 6-position of adenine and 4-position of cytosine. They were incorporated into DNA probes by standard nick translation protocols and probes labeled with biotin derivatives of these nucleotides were effectively hybridized to target DNA sequences. Thus, the modified forms of dATP and dCTP, when incorporated

into oligomers are recognized as A and C, respectively. Similarly, Gillam, I.C., et al., Anal Biochem (1986) 199-207, described the incorporation of N<sup>4</sup>-(6-aminohexyl) cytidine and deoxycytidine nucleotides into DNA enzymatically.

Trainor, G.L., et al., Nucleic Acids Res (1988) 16:11846, describe the ability of succinyl-fluorescein-labeled dideoxynucleoside triphosphates as substrates for terminal deoxynucleotidyl transferase and their use in the preparation of 3'-fluorescence-tagged DNA.

Mizusawa, S., et al., Nucleic Acids Res (1986) 14, described the replacement of dGTP in polymerase reactions by deoxy-7-deazaguanidine triphosphate; this is also described in the context of a PCR reaction by Innis, M.A., in "PCR Protocols: A Guide to Methods and Applications" (1990) Academic Press Inc.

The incorporation of 5-azido-dUTP appears to substitute for dTTP in polymerase reactions as reported by Evans, R.K., et al., Biochemistry (1987) 26:269-276; Proc Natl Acad Sci USA (1986) 83:5382-5386.

Oligonucleotides which contain covalently-bound mercury at specific base residues was described by Dale, R.M.K., et al., Proc Natl Acad Sci USA (1973) 70:2238-2242.

Finally, a terminal fluorescence residue using purines linked to fluorescing moieties is described by Prober, J.M., et al., Science 238:336.

Further, as set forth in the foregoing publications, not only is the modified base specifically recognized as such in a template sequence; nucleotide triphosphates utilizing the modified base are also capable of incorporation into the newly synthesized strand by polymerase enzymes.

Immune Recognition Mechanisms. This invention is also related to the use of specific binding oligomers

-11-

in immune recognition mechanisms. Various immune recognition mechanisms exist which permit recognition and immune destruction of malignant or infected cells in an organism. Malignant cells often express antigens that are not found in normal cells; some of these antigens are found at the surface of the cell. Similarly, pathogen-infected cells often express pathogen-encoded antigens at the cell surface. In both cases, the surface antigen represents a potential target for a CTL (cytotoxic T-cell) immune response.

Unfortunately, immune responses against unwanted cells are not always effective; moreover, such responses can, in some instances, be suppressed. A variety of mechanisms may play a role in the reduction or suppression of immune responses to pathologic cells. For example, tumors are associated with a decreased level of the histocompatibility antigens that may play a role in eliciting a CTL response. Viruses have also been able to mask viral antigens at the cell surface. In the case of HIV, heavy glycosylation of the envelope protein (normally found at the cell surface) may play a role in preventing an effective immune response against infected cells. The propensity of pathologic cells to reduce or escape effective CTL responses probably plays a role in the progression of various infections and disorders.

While some vaccines in current use consist of only portions of a pathogen (such as a viral envelope protein in the case of HBV virus), immune responses against an intact pathogen, such as a virus or bacterium, are often more effective than responses against individual components of the pathogen. Attenuated virus vaccines, for example, are used in some cases in order to expose the immune system to antigens that present epitopes in as natural a form as possible. The resulting immune response appears in general to result in

more effective protection against the pathogen than the corresponding response to only a portion of the pathogen.

Many CTL responses appear to be based upon specific contacts between a plurality of surface antigens serving as signals for both self and non-self cells. Normal immune function is believed to involve a combined response to this plurality of surface antigens. Hence, it is reasonable to expect that a modified immune response would result if one or more of these surface features were somehow modified or masked.

In addition to the use of antibodies specific for one or more of the subject surface features, considerable attention has also been directed recently to the use of oligonucleotides of similar specificity. De novo recovery of specifically binding oligonucleotides is possible with respect to non-oligonucleotide targets, as discussed above.

It would clearly be advantageous to devise methods which permit the modulation of immune response to natural antigens in a manner such that optimum immune protection against a pathogen or malignant cell may be obtained, or such that an undesired component of the response may be eliminated. In particular, it would be desirable to take advantage of the involvement of a plurality of epitopes in the normal immune response by developing immunomodulatory agents which target one or more specific epitopes involved in generating the immune response.

### Disclosure of the Invention

The invention described herein provides specifically binding oligonucleotides or "aptamers" that are stable, versatile, and highly specific for their intended targets. Furthermore, the aptamers of the invention may be determined as well as synthesized using

modified nucleotides and internucleotide linkages. In addition, these aptamers may be obtained from mixtures of candidate oligomers with completely unpredetermined sequences, without the necessity for inclusion of PCR primer sequences in the candidate pool. The efficiency of the method to determine suitable aptamers is further enhanced by separation of the complex containing successful candidate oligonucleotides bound to target from uncomplexed oligonucleotides and elution of the complex from solid support.

The aptamers of the present invention find a variety of utilities including therapeutic and diagnostic utilities as well as functioning as laboratory and industrial reagents. The aptamers of the invention can be coupled to various auxiliary substances such as label or solid support.

Thus, in one aspect, the invention is directed to an aptamer containing at least one binding region capable of binding specifically to a target molecule wherein the aptamer is a single-stranded DNA. Such single-stranded DNA aptamers can be constructed to bind specifically to a wide variety of target substances including proteins, peptides, glycoproteins, lipids, glycolipids, carbohydrates, and various small molecules. Such single-stranded DNA aptamers are advantageously stable as compared to RNA counterparts. It has been heretofore thought that the three-dimensional structure of double-stranded DNA limited the structural diversity of the molecule. The inventors herein are unaware of any prior demonstration of structural diversity for single- or double-stranded DNA sufficient to provide the range of conformations necessary to provide aptamers to biomolecules. For example, known RNA structures, such as pseudoknots, have not been described for single-stranded DNA.

In another aspect, the invention is directed to aptamers that have relatively short specific binding regions of less than 15 nucleotide residues and which may, themselves, be relatively small molecules containing less than 16 nucleotide residues. The limited length of these aptamers is advantageous in facilitating administration and synthesis. Further, in still other aspects, the invention is directed to aptamers with very low dissociation constants with respect to their target molecules (that do not normally bind oligonucleotides) of less than  $20 \times 10^{-9}$ ; and with high specificity for their targets of at least 5-fold differential in binding affinity as compared to competing substances. These enhanced specificities and binding affinities are clearly advantageous in the applications for which the aptamers of the invention are useful.

In another aspect, the invention is directed to aptamers that bind to a wide variety of target molecules, especially those selected from the group consisting of bradykinin, PGF2 $\alpha$ , CD4, HER2, IL-1 receptor, Factor X, and thrombin. The versatility of aptamers in specifically binding even small and hydrophobic molecules expands the range of their utility.

In other aspects, the invention is directed to complexes of the target molecules and the aptamers of the invention and to methods to obtain and to use the aptamers of the invention.

In still other aspects, the invention is directed to improved methods to obtain aptamers in general. These improved methods include the ability to utilize in a candidate pool of oligonucleotides completely undetermined sequences; to incorporate modified oligonucleotides in the candidate pool and to include modified nucleotides in the amplifying step of the method; to enhance the efficiency of the method by



isolating the complex between the successful members of the candidate pool and the target molecule; and to obtain aptamers that bind cell surface factors using a subtraction technique.

5 In still another aspect of the invention, aptamers may be used as specific binding agents in conjugates designed to modulate the immune system.

#### Brief Description of the Figures

10 Figure 1 is a chart depicting thrombin aptamer consensus-related sequences.

Figure 2 is a plot of in vivo thrombin inhibition obtained from primates using a 15-mer aptamer.

#### Modes of Carrying Out the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, biochemistry, protein chemistry, and recombinant DNA technology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Oligonucleotide Synthesis (M.J. Gait ed. 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins eds. 1984); Sambrook, Fritsch & Maniatis, Molecular Cloning: A  
20 Laboratory Manual, Second Edition (1989); and the series Methods in Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.).

The invention is directed to a method which permits the recovery and deduction of aptamers which bind  
30 specifically to desired targets including those illustrated hereinbelow such as factor X, kinins (including bradykinin) as well as other small peptide hormones such as the vasoconstrictor endothelin (a 21-mer peptide), small hydrophobic molecules such as  
35 eicosanoids (including PGF<sub>2</sub>α), and extracellular

proteins, such as thrombin, as well as molecules that are contained at the cell surface such as IL-1 receptor and CD4. As a result of application of this method, aptamers which contain the specifically binding sequences can be prepared and used in oligonucleotide-based therapy, in the detection and isolation of the target substance, as well as in other applications.

For example, these aptamers can be used as a separation tool for retrieving the targets to which they specifically bind. By coupling the oligonucleotides containing the specifically binding sequences to a solid support, for example, the target substances can be recovered in useful quantities. In addition, these oligonucleotides can be used in diagnosis by employing them in specific binding assays for the target substances. When suitably labeled using detectable moieties such as radioisotopes, the specifically binding oligonucleotides can also be used for in vivo imaging or histological analysis.

For application in such various uses, the aptamers of the invention may be coupled to auxiliary substances that enhance or complement the function of the aptamer. Such auxiliary substances include, for example, labels such as radioisotopes, fluorescent labels, enzyme labels and the like; specific binding reagents such as antibodies, additional aptamer sequence, cell surface receptor ligands, receptors per se and the like; toxins such as diphtheria toxin, tetanus toxin or ricin; drugs such as antiinflammatory, antibiotic, or metabolic regulator pharmaceuticals, solid supports such as chromatographic or electrophoretic supports, and the like. Suitable techniques for coupling of aptamers to desired auxiliary substances are generally known for a variety of such auxiliary substances, and the specific nature of the coupling procedure will depend on the

nature of the auxiliary substance chosen. Coupling may be direct covalent coupling or may involve the use of synthetic linkers such as those marketed by Pierce Chemical Co., Rockford, IL.

5           Thus, the aptamers of the invention may be used alone in therapeutic applications or may be used as targeting agents to deliver pharmaceuticals or toxins to desired targets. The aptamers may be used in diagnostic procedures and advantageously in this application include  
10 label. They may be used as reagents to separate target molecules from contaminants in samples containing the target molecules in which application they are advantageously coupled to solid support. A particularly advantageous application of the aptamers of the invention  
15 includes their use in an immune recruitment procedure as targeting agents for the immunomodulating substance used in this procedure, as further described below.

As used in the disclosure and claims, the following terms are defined as follows. All references  
20 cited are incorporated by reference.

As used herein, a "target" or "target molecule" refers to a biomolecule that could be the focus of a therapeutic drug strategy or diagnostic assay, including, without limitation, proteins or portions thereof,  
25 enzymes, peptides, enzyme inhibitors, hormones, carbohydrates, glycoproteins, lipids, phospholipids, nucleic acids, and generally, any biomolecule capable of turning a biochemical pathway on or off or modulating it, or which is involved in a predictable biological  
30 response. Targets may be free in solution, like thrombin, or associated with cells or viruses, as in receptors or envelope proteins.

It should be noted that excluded from target molecules are substances to which DNA sequences normally  
35 bind such as nucleases, substrates wherein binding is

effected by Watson-Crick base pairing modes of binding to nucleic acids, specific triple helix binding to nucleic acid sequences, and the like. Thus, excluded from target molecules are those substances which natively bind the specific form of aptamer at issue. Thus, excluded therefore are nucleases that attack single-stranded DNA, restriction endonucleases that attack double-stranded DNA with respect to single-stranded DNA and double-stranded DNA, respectively. Also excluded are cell surface receptors specific for DNA or RNA.

A wide variety of materials can serve as targets. These materials include intracellular, extracellular, and cell surface proteins, peptides, glycoproteins, carbohydrates, including glycosaminoglycans, lipids, including glycolipids and certain oligonucleotides. A representative list of targets for which the aptamers of the invention may be prepared is set forth herein in Table 1 which follows the examples in the herein specification.

Some of the useful targets are peptides such as kinins and small low molecular weight carbohydrates such as prostaglandins. These targets have particular features as follows:

By "kinin" is meant any of the peptide components enzymatically released by the activation of the various kininogens (hormogens). Thus, the term "kinin" includes the mammalian kinins such as, but not limited to, bradykinin (BK), Lys-BK, Met-Lys-BK, leukokinins, colostrokinin, neurokinin; the various nonmammalian kinins; and metabolites of the above. Kinins are small peptides having, on the average, 9-11 amino acids. As described above, there are several inherent problems associated with the use of conventional immunotechniques for working with kinins. Thus, the present invention provides an efficient method for the

detection and isolation of these important substances. For a review of kinins and their significance, see Regoli, D., and Barabe, J., Pharmacological Reviews (1980) 32:1-46, incorporated herein by reference in its entirety.

5 The subject invention is also useful for the detection and/or isolation of low molecular weight hydrophobic molecules. By "hydrophobic" is meant a compound having non-polar groups such that the compound  
10 as a whole has a relatively low affinity for water and other polar solvents. The hydrophobic molecules of the instant invention lack large numbers of groups that may participate in establishing noncovalent binding interactions with aptamers. Such interactions include  
15 base stacking via aromatic rings in the target, polar and ionic interactions, and hydrogen bonding.

The invention is particularly useful with fatty acid derivatives such as eicosanoids. By "eicosanoid" is meant any of the several members of the family of  
20 substances derived from 20-carbon essential fatty acids that contain three, four or five double bonds: 8,11,14-eicosatrienoic acid (dihomo- $\alpha$ -linolenic acid); 5,8,11,14-eicosatetraenoic acid (arachidonic acid) and 5,8,11,14,17-eicosapentaenoic acid. Such substances  
25 encompass the various prostaglandins, including but not limited to PGA, PGB, PGC, PGD, PGE, PGE1, PGE2, PGE2 $\alpha$ , PGF, PGF1 $\alpha$ , PGF2 $\alpha$ , PGG, PGG2, PGH, PGH2; the thromboxanes such as but not limited to TXA2 and TXB2; prostacyclin (PGI2) and 6-keto-PGF1 $\alpha$ ; leukotrienes and  
30 precursors thereof such as LTB4 (a 5,12-dihydroxy compound), LTC4 (a 5-hydroxy derivative that is conjugated with glutathione), LTA4 (a 5,6-epoxide), LTD4 (synthesized by the removal of glutamic acid from LTC4), LTE4 (resulting from the subsequent cleavage of glycine),  
35 LTF4 (an  $\alpha$ -glutamyl, cysteinyl derivative), SRS-A (a

mixture of LTC<sub>4</sub> and LTD<sub>4</sub> known as the "slow-reacting substance of anaphylaxis"), HPETE (hydroperoxyeicosatetraenoic acid) and HETE (monohydroxyeicosatetraenoic acid). Eicosanoids are also intended to include synthetic eicosanoid analogs such as 16-methoxy-16-methyl-PGF<sub>2</sub> $\alpha$  and 15-methyl-PGF<sub>2</sub> $\alpha$  (Guzzi, et al., J. Med. Chem. (1986) 29:1826-1832; Cheng, et al., Acta Acad. Med. Shanghai (1990) 17:378-381) or in vivo generated eicosanoid metabolites (Morrow, et al., Proc Natl. Acad. Sci. (USA) (1990) 87:9383-9387). Eicosanoids are relatively low molecular weight compounds which are generally hydrophobic in nature. These substances normally have molecular weights under 400, but some naturally occurring variants are conjugated to one or several amino acids and these will have higher molecular weights. These variants are also encompassed by the subject invention. As described above, several eicosanoids have not heretofore been easily detectable or isolatable using standard immunotechniques due to their ubiquitous nature. Thus, the present invention provides an efficient method for the detection and isolation of these important substances. For a review of eicosanoids and their significance, see Moncada, S., et al., in The Pharmacological Basis of Therapeutics, Gilman, A.G., et al., eds. (MacMillan Publishing Company, New York), 7th Edition, pages 660-671, incorporated herein by reference in its entirety.

The above small molecule and hydrophobic targets have not heretofore been considered to be potential target molecules for aptamer selection as oligonucleotides are very hydrophilic and highly hydrated. Previous methods for obtaining oligonucleotides that bind to targets utilized protein targets that normally bind to nucleic acids, or in the work described by Ellington, et al., Nature (1990)

346:818-822, target molecules with many possible hydrogen-bond donor and acceptor groups as well as planar surfaces for stacking interactions. In the case of nucleic acid binding proteins, binding to nucleic acid oligonucleotides is aided by the inherent binding properties of the proteins. In the case of molecules used by Ellington et al., numerous chemical structures are present that can participate in noncovalent binding interactions including planar aromatic rings that may interact with nucleic acids via base stacking interactions. In contrast, many eicosanoids such as PGF2 $\alpha$  have relatively little structural diversity. It is thus unexpected that fatty acid-like molecules may serve as binding targets for single stranded DNA. One representative eicosanoid, PGF2 $\alpha$ , as used in the present invention, has only 3 hydroxyl groups, two double bonds between adjacent methylene groups, a carboxylic acid group (which, as used herein, is present as an amide linkage for covalent attachment to a solid support) and a cyclopentyl ring. By comparison with almost all other classes of potential biological target molecules, the eicosanoids are extremely deficient in groups that may participate in noncovalent binding interactions.

As used herein, "specifically binding oligonucleotides" or "aptamers" refers to oligonucleotides having specific binding regions which are capable of forming complexes with an intended target molecule in an environment wherein other substances in the same environment are not complexed to the oligonucleotide. The specificity of the binding is defined in terms of the comparative dissociation constants ( $K_d$ ) of the aptamer for target as compared to the dissociation constant with respect to the aptamer and other materials in the environment or unrelated molecules in general. Typically, the  $K_d$  for the aptamer with

respect to the target will be 2-fold, preferably 5-fold, more preferably 10-fold less than  $K_d$  with respect to target and the unrelated material or accompanying material in the environment. Even more preferably the  $K_d$  will be 50-fold less, more preferably 100-fold less, and more preferably 200-fold less.

The binding affinity of the aptamers herein with respect to targets and other molecules is defined in terms of  $K_d$ . The value of this dissociation constant can be determined directly by well-known methods, and can be computed even for complex mixtures by methods such as those, for example, set forth in Caceci, M., et al., Byte (1984) 2:340-362. It has been observed, however, that for some small oligonucleotides, direct determination of  $K_d$  is difficult, and can lead to misleadingly high results. Under these circumstances, a competitive binding assay for the target molecule or other candidate substance may be conducted with respect to substances known to bind the target or candidate. The value of the concentration at which 50% inhibition occurs ( $K_i$ ) is, under ideal conditions, equivalent to  $K_d$ . However, in no event can  $K_i$  be less than  $K_d$ . Thus, determination of  $K_i$ , in the alternative, sets a maximal value for the value of  $K_d$ . Under those circumstances where technical difficulties preclude accurate measurement of  $K_d$ , measurement of  $K_i$  can conveniently be substituted to provide an upper limit for  $K_d$ .

As specificity is defined in terms of  $K_d$  as set forth above, excluded from the categories of unrelated materials and materials accompanying the target in the target's environment are those materials which are sufficiently related to the target to be immunologically crossreactive therewith, and materials which natively bind oligonucleotides of particular sequences such as nucleases, restriction enzymes, and the like. By



"immunologically crossreactive" is meant that antibodies raised with respect to the target crossreact under standard assay conditions with the candidate material. Generally, for antibodies to crossreact in standard  
5 assays, the binding affinities of the antibodies for crossreactive materials as compared to targets should be in the range of 5-fold to 100-fold, generally about 10-fold.

Thus, aptamers which contain specific binding  
10 regions are specific with respect to unrelated materials and with respect to materials which do not normally bind such oligonucleotides such as nucleases and restriction enzymes.

In general, a minimum of approximately 6  
15 nucleotides, preferably 10, and more preferably 14 or 15 nucleotides, are necessary to effect specific binding. The only apparent limitations on the binding specificity of the target/oligonucleotide couples of the invention concern sufficient sequence to be distinctive in the  
20 binding oligonucleotide and sufficient binding capacity of the target substance to obtain the necessary interaction. Aptamers of binding regions containing sequences shorter than 10, e.g., 6-mers, are feasible if the appropriate interaction can be obtained in the  
25 context of the environment in which the target is placed. Thus, if there are few interferences by other materials, less specificity and less strength of binding may be required.

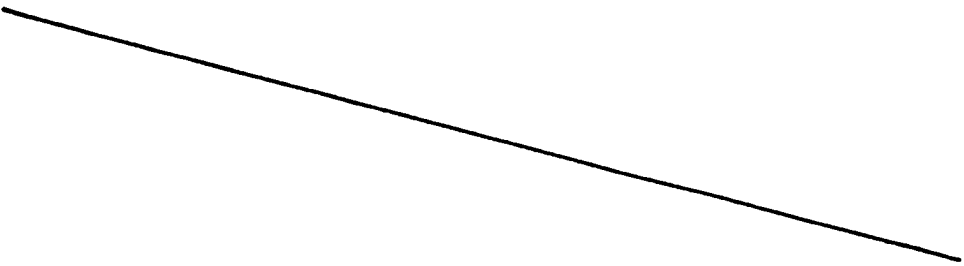
As used herein, "aptamer" refers in general to  
30 either an oligonucleotide of a single defined sequence or a mixture of said oligonucleotides, wherein the mixture retains the properties of binding specifically to the target molecule. Thus, as used herein "aptamer" denotes both singular and plural sequences of oligonucleotides,  
35 as defined hereinabove.

Structurally, the aptamers of the invention are specifically binding oligonucleotides, wherein "oligonucleotide" is as defined herein. As set forth herein, oligonucleotides include not only those with  
5 conventional bases, sugar residues and internucleotide linkages, but also those which contain modifications of any or all of these three moieties.

"Single-stranded" oligonucleotides, as the term is used herein, refers to those oligonucleotides which  
10 contain a single covalently linked series of nucleotide residues.

"Oligomers" or "oligonucleotides" include RNA or DNA sequences of more than one nucleotide in either single chain or duplex form and specifically includes  
15 short sequences such as dimers and trimers, in either single chain or duplex form, which may be intermediates in the production of the specifically binding oligonucleotides.

"Oligonucleotide" or "oligomer" is generic to  
20 polydeoxyribonucleotides (containing 2'-deoxy-D-ribose or modified forms thereof), i.e., DNA, to polyribonucleotides (containing D-ribose or modified forms thereof), i.e., RNA, and to any other type of polynucleotide which is an N-glycoside or C-glycoside of a purine or  
25 pyrimidine base, or modified purine or pyrimidine base or abasic nucleotides.



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The oligomers of the invention may be formed using conventional phosphodiester-linked nucleotides and synthesized using standard solid phase (or solution phase) oligonucleotide synthesis techniques, which are now commercially available. However, the oligomers of the invention may also contain one or more "substitute" linkages as is generally understood in the art. Some of these substitute linkages are non-polar and contribute to the desired ability of the oligomer to diffuse across membranes. These "substitute" linkages are defined herein as conventional alternative linkages such as phosphorothioate or phosphoramidate, are synthesized as described in the generally available literature. Alternative linking groups include, but are not limited to embodiments wherein a moiety of the formula  $P(O)S$ , ("thioate"),  $P(S)S$  ("dithioate"),  $P(O)NR'_2$ ,  $P(O)R'$ ,  $P(O)OR^6$ ,  $CO$ , or  $CONR'_2$ , wherein  $R'$  is H (or a salt) or alkyl (1-12C) and  $R^6$  is alkyl (1-9C) is joined to adjacent nucleotides through -O- or -S-. Dithioate linkages are disclosed and claimed in commonly owned U.S. application no. 248,517. Substitute linkages that may be used in the oligomers disclosed herein also include nonphosphorous-based internucleotide linkages such as the 3'-thioformacetal (-S-CH<sub>2</sub>-O-), formacetal (-O-CH<sub>2</sub>-O-) and 3'-amine (-NH-CH<sub>2</sub>-CH<sub>2</sub>-) internucleotide linkages disclosed and claimed in commonly owned pending U.S. patent application serial nos. 690,786 and 763,130, both incorporated herein by reference. One or more substitute linkages may be utilized in the oligomers in order to further facilitate binding with complementary target nucleic acid sequences or to increase the stability of the oligomers toward nucleases, as well as to confer permeation ability. (Not all such linkages in the same oligomer need be identical.)

The term "nucleoside" or "nucleotide" is similarly generic to ribonucleosides or ribonucleotides, deoxyribonucleosides or deoxyribonucleotides, or to any other nucleoside which is an N-glycoside or C-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base. Thus, the stereochemistry of the sugar carbons may be other than that of D-ribose in one or more residues. Also included are analogs where the ribose or deoxyribose moiety is replaced by an alternate structure such as the 6-membered morpholino ring described in U.S. patent number 5,034,506 or where an acyclic structure serves as a scaffold that positions the base analogs described herein in a manner that permits efficient binding to target nucleic acid sequences or other targets. Elements ordinarily found in oligomers, such as the furanose ring or the phosphodiester linkage may be replaced with any suitable functionally equivalent element. As the  $\alpha$  anomer binds to targets in a manner similar to that for the  $\beta$  anomers, one or more nucleotides may contain this linkage or a domain thereof. (Praseuth, D., et al., Proc Natl Acad Sci (USA) (1988) 85:1349-1353). Modifications in the sugar moiety, for example, wherein one or more of the hydroxyl groups are replaced with halogen, aliphatic groups, or functionalized as ethers, amines, and the like, are also included.

"Nucleoside" and "nucleotide" include those moieties which contain not only the natively found purine and pyrimidine bases A, T, C, G and U, but also modified or analogous forms thereof. Modifications include alkylated purines or pyrimidines, acylated purines or pyrimidines, or other heterocycles. Such "analogous purines" and "analogous pyrimidines" are those generally known in the art, many of which are used as chemotherapeutic agents. An exemplary but not exhaustive

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list includes pseudoisocytosine,  $N^4, N^4$ -ethanocytosine, 8-hydroxy- $N^6$ -methyladenine, 4-acetylcytosine, \*5-(carboxyhydroxymethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyl uracil, dihydrouracil, inosine,  $N^6$ -isopentenyl-adenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine,  $N^6$ -methyladenine, 7-methylguanine, 5-methylaminomethyl uracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio- $N^6$ -isopentenyladenine, uracil-5-oxyacetic acid methyl ester, pseudouracil, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, queosine, 2-thiocytosine, 5-propyluracil, 5-propylcytosine, 5-ethyluracil, 5-ethylcytosine, 5-butyluracil, 5-butylcytosine, 5-pentyluracil, 5-pentylcytosine, and 2,6-diaminopurine.

In addition to the modified bases above, nucleotide residues which are devoid of a purine or a pyrimidine base may also be included in the aptamers of the invention and in the methods for their obtention.

The sugar residues in the oligonucleotides of the invention may also be other than conventional ribose and deoxyribose residues. In particular, substitution at the 2'-position of the furanose residue is particularly important.

Aptamer oligonucleotides may contain analogous forms of ribose or deoxyribose sugars that are generally known in the art. An exemplary, but not exhaustive list includes 2' substituted sugars such as 2'-O-methyl-, 2'-O-alkyl, 2'-O-allyl, 2'-S-alkyl, 2'-S-allyl, 2'-fluoro-, 2'-halo, or 2'-azido-ribose, carbocyclic sugar analogs,

5  $\alpha$ -anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside, ethyl riboside or propyl riboside.

Although the conventional sugars and bases will be used in applying the method of the invention, substitution of analogous forms of sugars, purines and pyrimidines can be advantageous in designing the final product. Additional techniques, such as methods of synthesis of 2'-modified sugars or carbocyclic sugar analogs, are described in Sproat, B.S. et al., Nucl Acid Res (1991) 19:733-738; Cotten, M. et al., Nuc Acid Res (1991) 19:2629-2635; Hobbs, J. et al., Biochemistry (1973) 12:5138-5145; and Perbost, M. et al., Biochem Biophys Res Comm (1989) 165:742-747 (carbocyclics).

As used herein, "primer" refers to a sequence which is capable of serving as an initiator molecule for a DNA polymerase when bound to complementary DNA which is usually between 3-25 nucleotides in length.

As used herein, a "type II restriction enzyme site" refers to a site possessed by the class of restriction enzymes which cleaves one or both DNA strands at internucleotide linkages that are located outside of those associated with bases in the recognition sequence. This term is also meant herein to refer to a restriction enzyme such as Bcg I (New England Biolabs, catalog no. 545L) that makes two double stranded DNA cuts outside of its recognition sequence.

One of the objects of the invention is to identify aptamers useful as drugs per se or useful in drug development. Toward this end, selection criteria for targets and aptamers include:

1. The aptamer should selectively bind to the desired target, thereby inhibiting a biochemical pathway

or generating a specific response (e.g., modulating an immune response or disrupting binding interactions between a receptor and its ligand);

2. The aptamer selected for use in diagnostic applications should have specificity for analyte (ligand) binding in those cases where the aptamer will be immobilized to a support;

3. The biochemical pathway that is inhibited or the biological response generated should be related to a pathological disease state in such a way that inhibition of that pathway or the biological response generated in a patient is therapeutic;

4. Desirably, the aptamer is specific so that it does not appreciably inhibit other pathways or generate additional unwanted biological responses;

5. Preferred aptamers have or are capable of being adapted to have the pharmacokinetic characteristics of a practical drug (i.e., they must be absorbed, must penetrate to the site of action and must have a reasonably predictable dose response relationship and duration of action);

6. Desirably, the aptamer has an acceptable toxicological profile in animals and the results of human clinical trials must demonstrate an appropriate therapeutic use.

#### Methods to Prepare the Invention Aptamers

In general, the method for preparing the aptamers of the invention involves incubating a desired target molecule with a mixture of oligonucleotides under conditions wherein some but not all of the members of the oligonucleotide mixture form complexes with the target molecules. The resulting complexes are then separated from the uncomplexed members of the oligonucleotide mixture and the complexed members which constitute an

aptamer (at this stage the aptamer generally being a population of a multiplicity of oligonucleotide sequences) is recovered from the complex and amplified. The resulting aptamer (mixture) may then be substituted for the starting mixture in repeated iterations of this series of steps. When satisfactory specificity is obtained, the aptamer may be used as a obtained or may be sequenced and synthetic forms of the aptamer prepared. In this most generalized form of the method, the oligonucleotides used as members of the starting mixture may be single-stranded or double-stranded DNA or RNA, or modified forms thereof. However, single-stranded DNA is preferred. The use of DNA eliminates the need for conversion of RNA aptamers to DNA by reverse transcriptase prior to PCR amplification. Furthermore, DNA is less susceptible to nuclease degradation than RNA.

The oligonucleotides that bind to the target are separated from the rest of the mixture and recovered and amplified. Amplification may be conducted before or after separation from the target molecule. The oligonucleotides are conveniently amplified by PCR to give a pool of DNA sequences. The PCR method is well known in the art and described in, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202 and Saiki, R.K., et al., Science (1988) 239:487-491, and European patent applications 86302298.4, 86302299.2 and 87300203.4, as well as Methods in Enzymology (1987) 155:335-350. If RNA is initially used, the amplified DNA sequences are transcribed into RNA. The recovered DNA or RNA, in the original single-stranded or duplex form, is then used in another round of selection and amplification. After three to six rounds of selection/amplification, oligomers that bind with an affinity in the mM to  $\mu$ M range can be obtained for most targets and affinities below the  $\mu$ M



range are possible for some targets. PCR may also be performed in the presence of target.

Other methods of amplification may be employed including standard cloning, ligase chain reaction, etc. (See e.g., Chu, et al., U.S. Patent No. 4,957,858). For example, to practice this invention using cloning, once the aptamer has been identified, linkers may be attached to each side to facilitate cloning into standard vectors. Aptamers, either in single or double stranded form, may be cloned and recovered thereby providing an alternative amplification method.

Amplified sequences can be applied to sequencing gels after any round to determine the nature of the aptamers being selected by target molecules. The entire process then may be repeated using the recovered and amplified duplex if sufficient resolution is not obtained.

Amplified sequences can be cloned and individual oligonucleotides then sequenced. The entire process can then be repeated using the recovered and amplified oligomers as needed. Once an aptamer that binds specifically to a target has been selected, it may be recovered as DNA or RNA in single-stranded or duplex form using conventional techniques.

Similarly, a selected aptamer may be sequenced and resynthesized using one or more modified bases, sugars and linkages using conventional techniques. The specifically binding oligonucleotides need to contain the sequence-conferring specificity, but may be extended with flanking regions and otherwise derivatized.

The starting mixture of oligonucleotide may be of undetermined sequence or may preferably contain a randomized portion, generally including from about 3 to about 400 nucleotides, more preferably 10 to 100 nucleotides. The randomization may be complete, or there

may be a preponderance of certain sequences in the mixture, or a preponderance of certain residues at particular positions. Although, as described hereinbelow, it is not essential, the randomized sequence is preferably flanked by primer sequences which permit the application of the polymerase chain reaction directly to the recovered oligonucleotide from the complex. The flanking sequences may also contain other convenient features, such as restriction sites which permit the cloning of the amplified sequence. These primer hybridization regions generally contain 10 to 30, more preferably 15 to 25, and most preferably 18 to 20, bases of known sequence.

The oligonucleotides of the starting mixture may be conventional oligonucleotides, most preferably single-stranded DNA, or may be modified forms of these conventional oligomers as described hereinabove. For oligonucleotides containing conventional phosphodiester linkages or closely related forms thereof, standard oligonucleotide synthesis techniques may be employed. Such techniques are well known in the art, such methods being described, for example, in Froehler, B., et al., Nucleic Acids Research (1986) 14:5399-5467; Nucleic Acids Research (1988) 16:4831-4839; Nucleosides and Nucleotides (1987) 6:287-291; Froehler, B., Tet Lett (1986) 27:5575-5578. Oligonucleotides may also be synthesized using solution phase methods such as triester synthesis, known in the art. The nature of the mixture is determined by the manner of the conduct of synthesis. Randomization can be achieved, if desired, by supplying mixtures of nucleotides for the positions at which randomization is desired. Any proportion of nucleotides and any desired number of such nucleotides can be supplied at any particular step. Thus, any degree of randomization may be employed. Some positions may be randomized by

mixtures of only two or three bases rather than the conventional four. Randomized positions may alternate with those which have been specified. It may be helpful if some portions of the candidate randomized sequence are  
5 in fact known.

In one embodiment of the method of the invention, the starting mixture of oligonucleotides subjected to the invention method will have a binding affinity for the target characterized by a  $K_d$  of 1  $\mu M$  or greater. Binding affinities of the original mixture for  
10 target may range from about 100  $\mu M$  to 10  $\mu M$  to 1  $\mu M$ , but, of course, the smaller the value of the dissociation constant, the more initial affinity there is in the starting material for the target. This may or may not be  
15 advantageous as specificity may be sacrificed by starting the procedure with materials with high binding affinity.

By application of the method of the invention as described herein, improvements in the binding affinity over one or several iterations of the above steps of at  
20 least a factor of 50, preferably of a factor of 100, and more preferably of a factor of 200 may be achieved. As defined herein, a ratio of binding affinity reflects the ratio of  $K_d$ s of the comparative complexes. Even more preferred in the conduct of the method of the invention  
25 is the achievement of an enhancement of an affinity of a factor of 500 or more.

Thus, the method of the invention can be conducted to obtain the invention aptamers wherein the aptamers are characterized by consisting of single-  
30 stranded DNA, or by having a binding affinity to a target that does not normally bind oligonucleotides represented by a  $K_d$  of  $20 \times 10^{-9}$  or less, or by having a specificity representing by a factor of at least 2, preferably 5, and more preferably 10 with respect to unrelated molecules,  
35 or by having a binding region of less than 15 nucleotide

residues or a total size of less than 16 nucleotide residues, or by binding to particular target molecules. The invention processes are also characterized by accommodating starting mixtures of oligonucleotides having a binding affinity for target characterized by a  $K_d$  of 1  $\mu$ M or more by an enhancement of binding affinity of 50 or more, and by being conducted under physiological conditions.

As used herein, physiological conditions means the salt concentration and ionic strength in an aqueous solution which characterize fluids found in human metabolism commonly referred to as physiological buffer or physiological saline. In general, these are represented by an intracellular pH of 7.1 and salt concentrations (in mM) of  $\text{Na}^+$ : 3-15;  $\text{K}^+$ : 140;  $\text{Mg}^{+2}$ : 6.3;  $\text{Ca}^{+2}$ :  $10^{-4}$ ;  $\text{Cl}^-$ : 3-15, and an extracellular pH of 7.4 and salt concentrations (in mM) of  $\text{Na}^+$ : 145;  $\text{K}^+$ : 3;  $\text{Mg}^{+2}$ : 1-2;  $\text{Ca}^{+2}$ : 1-2; and  $\text{Cl}^-$ : 110.

The use of physiological conditions in the aptamer selection method is extremely important, particularly with respect to those aptamers that may be intended for therapeutic use. As is understood in the art, the concentration of various ions, in particular, the ionic strength, and the pH value impact on the value of the dissociation constant of the target/aptamer complex.

#### Use of Modified Nucleotides and Oligonucleotides

In one embodiment of the invention method, the initial mixture of candidate oligonucleotides will include oligomers which contain at least one modified nucleotide residue or linking group.

If certain specific modifications are included in the amplification process as well, advantage can be taken of additional properties of any modified

nucleotides, such as the presence of specific affinity agents in the purification of the desired materials.

In order for the modified oligomer to yield useful results, the modification must result in a residue which is "read" in a known way by the polymerizing enzyme used in the amplification procedure. It is not necessary that the modified residue be incorporated into the oligomers in the amplification process, as long it is possible to discern from the nucleotide incorporated at the corresponding position the nature of the modification contained in the candidate, and provided only one round of complexation/amplification is needed. However, many of the modified residues of the invention are also susceptible to enzymatic incorporation into oligonucleotides by the commonly used polymerase enzymes and the resulting oligomers will then directly read on the nature of the candidate actually contained in the initial complex. It should be noted that if more than one round of complexation is needed, the amplified sequence must include the modified residue, unless the entire pool is sequenced and resynthesized to include the modified residue.

Certain modifications can be made to the base residues in a oligonucleotide sequence without impairing the function of polymerizing enzymes to recognize the modified base in the template or to incorporate the modified residue. These modifications include alkylation of the 5-position of uridine, deoxyuridine, cytidine and deoxycytidine; the N<sup>4</sup>-position of cytidine and deoxycytidine; the N<sup>6</sup>-position of adenine and deoxyadenine; the 7-position of 7-deazaguanine, 7-deaza-deoxyguanine, 7-deazaadenine and 7-deazadeoxyadenine. As long as the nature of the recognition is known, the modified base may be included in the oligomeric mixtures useful in the method of the invention.

The nature of the sugar moiety may also be modified without affecting the capacity of the sequence to be usable as a specific template in the synthesis of new DNA or RNA.

5           The efficacy of the process of selection and amplification depends on the ability of the PCR reaction faithfully to reproduce the sequence actually complexed to the target substance. Thus, if the target substance contains modified forms of cytosine (C\*), the PCR  
10 reaction must recognize this as a modified cytosine and yield an oligomer in the cloned and sequenced product which reflect this characterization. If the modified form of cytosine (C\*) is included in the PCR reaction as dC\*TP, the resulting mixture will contain C\* at positions  
15 represented by this residue in the original member of the candidate mixture. (It is seen that the PCR reaction cannot distinguish between various locations of C\* in the original candidate; all C residue locations will appear as C\*.) Conversely, dCTP could be used in the PCR  
20 reaction and it would be understood that one or more of the positions now occupied by C was occupied in the original candidate mixture by C\*, provided only one round of complexation/amplification is needed. If the amplified mixture is used in a second round, this new  
25 mixture must contain the modification.

Of course, if the selected aptamer is sequenced and resynthesized, modified oligonucleotides and linking groups may arbitrarily be used in the synthesized form of the aptamer.

30           Inclusion of modified oligonucleotides in the methods and aptamers of the invention provides a tool for expansion of the repertoire of candidates to include large numbers of additional oligonucleotide sequences. Such expansion of the candidate pool may be especially  
35 important as the demonstration of binding to proteins,

for example, in the prior art is limited to those proteins known to have the capability to bind DNA. Modifications of the oligonucleotide may be necessary to include all desired substances among those targets for which specific binding can be achieved.

Thus, one preferred method comprises incubating the target with a mixture of oligonucleotides, wherein these oligonucleotides contain at least one modified nucleotide residue or linkage, under conditions wherein complexation occurs with some but not all members of the mixture; separating the complexed from uncomplexed oligonucleotides, recovering and amplifying the complexed oligonucleotides and optionally determining the sequence of the recovered nucleotides. In an additional preferred embodiment, amplification is also conducted in the presence of modified nucleotides.

#### Use of Starting Oligonucleotide Mixtures of Unpredetermined Sequence

In another embodiment, a method for making aptamers is provided, based on the discovery that the presence of flanking sequences (usually primer binding sequences) on the oligonucleotides of the candidate mixture may limit aptamer structural diversity and/or inhibit binding, thereby resulting in less than the full range of structural variation that is possible in a given pool of aptamers. This embodiment may use mixtures of unbiased oligonucleotide pools, and provides the ability to then engineer appropriate means for amplifying the desired oligonucleotides (putative aptamers).

Once single stranded aptamers are generated, linkers may be added to both ends as described herein (much in the same manner as a sticky end ligation). Preferably the linkers are partially double stranded and have some overhang to and at both ends to facilitate

cloning into a standard cloning vector. One of the overhangs should be a random sequence to provide complementarity to permit binding to the aptamer. The other overhang may provide necessary bases for sticky end

5 ligation.

In one embodiment the method comprises:

(a) providing a mixture of oligonucleotides of unknown, non-predetermined or substantially non-predetermined, said mixture comprising a quantity of  
10 oligonucleotides sufficiently reflective of the structural complexity of said target as to statistically ensure the presence of at least one oligonucleotide capable of binding said target;

(b) incubating said mixture of oligonucleotides  
15 with said target under conditions wherein complexation occurs between some oligonucleotides and said target, said complexed oligonucleotides defining an aptamer population;

(c) recovering said aptamers in substantially  
20 single stranded form;

(d) attaching a known nucleotide sequence to at least one end of said aptamers;

(e) amplifying said aptamers; and

(f) removing said known nucleotide sequence  
25 from said aptamers.

In the first step, the oligonucleotides comprising the mixture may be of completely unknown sequence. The oligonucleotides comprising the pool also may be of partially known sequence, but without flanking  
30 primer regions. The invention is not limited to first generation aptamers, but may be practiced to identify second and third generation aptamers as well. Oligonucleotides comprising the pool from which second and third generation aptamers may be identified, may



have, for example, 40%-70% of their sequences known or predetermined.

One skilled in the art will recognize that the diversity of the oligonucleotide pool from which aptamers are identified may be reduced, either by using known sequences, or through the processes of retention and selection by which these aptamers are made. As pool size and pool diversity is reduced, more aptamers capable of more specific binding are recovered. Stated in another way, the quantity of oligonucleotides in the pool and the diversity and/or complexity of the pool are inversely related.

These aspects of the invention are elucidated in the following embodiment which adds additional steps to steps (a)-(f) listed above:

(g) repeating steps a-f using said first aptamers of step (f), or a portion thereof, to comprise a second pool of oligonucleotides for use in step (a), thereby generating a second aptamer population which may be used to repeat steps (a)-(f), and optionally

(h) repeating steps (a)-(f) using said second aptamers of step (g), or a portion thereof, a sufficient number of times so as to identify an optimal aptamer population from which at least one consensus region may be identified in at least two of the aptamers from said optimal aptamer population, the presence of which may be correlated with aptamer to target binding or to aptamer structure.

This method includes methods for selectively attaching and removing flanking regions to aptamers, thereby permitting aptamer recovery in high yield. One such method comprises,

after separating oligonucleotides in the method above in substantially single stranded form from the pool capable of binding target;

attaching a 5' linker of known sequence to a first (the 5') end of the oligonucleotides, the 5' linker having a first type II restriction enzyme recognition site at its 3' end, attaching a 3' linker of known sequence to a second (the 3') end of the oligonucleotides, the 3' linker having a second type II restriction enzyme recognition site different from the site at the 5' end;

amplifying the oligonucleotides, thereby generating a duplex comprising a first (upper) strand, having a 5' linker complement portion, an oligonucleotide complement portion and a 3' linker complement portion, and a second (lower) strand, comprising a 5' linker portion, an oligonucleotide portion and a 3' linker portion;

removing the 5' and the 3' linker portions from the oligonucleotides; and

recovering the oligonucleotides in substantially single stranded form.

Another method of effecting amplification comprises,

after recovering oligonucleotides from the above bound pool in substantially single stranded form;

attaching a double stranded DNA linker of known sequence having at least 2-4 bases of random sequence present as a 3' overhang, said 2-4 bases capable of hybridizing to the 3' end of said oligonucleotides, the linker having a first type II restriction enzyme recognition site;

attaching a double stranded DNA linker of known sequence having at least 2-4 bases of random sequence present as a 3' overhang, the 2-4 bases capable of hybridizing to the 5' end of the oligonucleotides, said linker having a second type II restriction enzyme recognition site;

amplifying said oligonucleotides, thereby generating duplexes comprising a first (upper) strand, having a 5' linker complement portion, an oligonucleotide complement portion and a 3' linker complement portion, and a second (lower) strand, comprising a 5' linker portion, an oligonucleotide portion and a 3' linker portion;

removing the 3' linker portion from the oligonucleotide by attaching the product of step 4 above to a solid support, removing the 3' linker by digesting with a type II restriction enzyme capable of recognizing said first type II restriction enzyme binding site, removing the 5' linker complement and the oligonucleotide complement by heat denaturation, annealing a 5' linker complement to the upper strand, and removing the 5' linker portion by digesting with a type II restriction enzyme capable of recognizing the second type II restriction enzyme site; and

recovering the oligonucleotides in substantially single stranded form.

In another approach, the method includes attaching a single RNA residue to the 5' linker portion and removing it after amplification by cleaving the RNA linkage.

25

#### A Subtraction Method for Aptamer Preparation

It is often advantageous in enhancing the specificity of the aptamer obtained to remove members of the starting oligonucleotide mixture which bind to a second substance from which the target molecule is to be distinguished. This method is particularly useful in obtaining aptamers which bind to targets that reside on cell surfaces since a large number of contaminating materials will surround the desired target. In such subtraction methods, at least two rounds of selection and

35

amplification will be conducted. In a positive/negative selection approach, the target will be incubated with the starting mixture of oligonucleotides and, as usual, the complexes formed are separated from uncomplexed  
5 oligonucleotides. The complexed oligonucleotides, which are now an aptamer, are recovered and amplified from the complex. The recovered aptamer is then mixed with the second, undesired, substance from which the target is to be distinguished under conditions wherein members of the  
10 aptamer population which bind to said second substance can be complexed. This complex is then separated from the remaining oligonucleotides of the aptamer. The resulting unbound second aptamer population is then recovered and amplified. The second aptamer population  
15 is highly specific for the target as compared to the second substance.

In an alternative approach, the negative selection step may be conducted first, thus mixing the original oligonucleotide mixture with the undesired  
20 substance to complex away the members of the oligonucleotide mixture which bind to the second substance; the uncomplexed oligonucleotides are then recovered and amplified and incubated with the target under conditions wherein those members of the  
25 oligonucleotide mixture which bind targets are complexed. The resulting complexes then removed from the uncomplexed oligonucleotides and the bound aptamer population is recovered and amplified as usual.

When applied to the preparation of aptamers  
30 which bind specifically targets residing on cell surfaces, the positive round is conducted preferably with the target expressed at the surface of a cell, said expression typically occurring through recombinant transformation or by virtue of the native properties of  
35 the cell. The negative round of selection is conducted

with similar cells which have similar surface materials associated with them, but which do not express the desired target.

The methods and aptamers of the present invention can also be directed to cell surface proteins. To prepare these aptamers, a pool of oligonucleotides is brought into contact with a first known cell line which is known to express a particular cell surface protein which is uniquely identified with that cell line and sufficient time is allowed for the oligonucleotides to bind to the protein on the cell surfaces. The cells are isolated with oligonucleotides bound thereto and the oligonucleotides are removed. This procedure is referred to herein as "positive screening". Thereafter, the removed oligonucleotides are brought into contact with a second cell line which is identical to the first cell line, except that the second cell line does not express the particular identifying cell surface protein; binding is allowed to occur and any oligonucleotides which bind to the second cell line are isolated and discarded. This procedure is referred to as "negative screening". The "positive" and "negative" screening steps can be repeated a multiplicity of times in order to obtain oligonucleotides which are highly specific for the cell surface proteins being expressed on the first cell line. The highly specific oligonucleotides may then be amplified and sequenced.

A preferred variation for selection of aptamers that bind to surface antigens involves a procedure wherein negative selection is first carried out followed by a positive selection. In accordance with this procedure, a pool of random oligonucleotides is combined with a tissue culture medium. The oligonucleotides are allowed to remain in contact with the cell cultures for a sufficient period of time to allow binding between

oligonucleotides and cell surfaces which lack the target molecule. When this binding occurs, a negative selection process has been carried out, i.e., oligonucleotides which are not the desired aptamers can be eliminated by  
5 their binding to nontarget surfaces. Following this negative selection, a positive selection step is carried out. This is done by combining the oligonucleotides which did not bind to the surfaces lacking target molecules thereon with a cell culture containing the  
10 target molecule on their surface. Such a negative-positive selection protocol can be carried out in a medium containing human or bovine serum in order to select aptamers under simulated physiological conditions. It is desirable to replicate physiological conditions as  
15 closely as possible when carrying out the selection processes in that one endeavors to find oligonucleotides (aptamers) which bind to the target molecules under physiological conditions so that such aptamers can later be used in vivo.

20 In more detail, the oligonucleotide mixture is brought into contact with a first known cell line which is known to express a particular cell surface protein which is uniquely identified with that cell line. After allowing sufficient time for the oligonucleotides to bind  
25 to the protein on the cell surfaces, procedures are carried out to isolate the cells with oligonucleotides bound thereto and the oligonucleotides are removed. This procedure is referred to herein as "positive screening".

After treatments with the candidate  
30 oligonucleotide mixtures, the cells containing the targeted surface protein may be extensively washed in buffered saline or in tissue culture medium to remove low affinity aptamers and uncomplexed oligonucleotides. Following washing, the cells are treated with one or more  
35 of a number of agents that permit recovery of bound

aptamers. The cells may be treated enzymatically with trypsin or other proteases to cleave the targets at the cell surface, thus releasing the bound aptamers. Alternatively, the cells containing bound aptamers may be washed in a detergent or high ionic strength solution in order to disrupt binding between the cells and aptamers. The aptamers recovered at this point consist of a pool of different sequences that bind to different cell surface targets, including the target of interest.

10 Aptamers from the first tissue culture cells may be recovered from solution by precipitation or may be used directly if reagents used to remove aptamers do not significantly affect cells in the second tissue culture.

15 The aptamer mixture is then incubated with the second (null) cell culture under similar conditions. The mixture brought into contact with a second cell line which is identical to the first cell line, except that the second cell line does not express the particular identifying cell surface protein. Binding is allowed to occur and any oligonucleotides which bind to the second cell line are isolated and discarded. This procedure is referred to as "negative screening". The "positive" and "negative" screening steps can be repeated a multiplicity of times in order to obtain oligonucleotides which are highly specific for the cell surface proteins being expressed on the first cell line. When the highly specific oligonucleotides have been determined and isolated, they are subjected to PCR technology for amplification as above. The resulting "aptamers" can be labeled and thereafter effectively used to identify the presence of the first cell line expressing the particular cell surface protein.

35 This method identifies target features on cell surfaces such as proteins, especially hetero- or homodimers or multimers. Selecting high-affinity ligands

specific for such transmembrane proteins outside the natural cellular context has heretofore been exceedingly difficult, if not impossible. Many transmembrane proteins cannot be isolated from cells without loss of their native structure and function. This is due, in part, to a requirement for detergents to disrupt cellular membranes that anchor transmembrane proteins (Helenius, A., et al., Biochim. Biophys. Acta (1975) 415:27-79). Detergents that solubilize membranes also tend to denature proteins, leading to loss of function and alteration of native structure.

A preferred variation of this method involves a procedure wherein negative selection is first carried out followed by a positive selection. In accordance with this procedure, a pool of random oligonucleotides is combined with a tissue culture medium. The oligonucleotides are allowed to remain in contact with the cell cultures for a sufficient period of time to allow binding between oligonucleotides and cell surfaces which lack the target molecule. When this binding occurs, a negative selection process has been carried out, i.e., oligonucleotides which are not the desired aptamers can be eliminated by their binding to nontarget surfaces. Following this negative selection, a positive selection step is carried out. This is done by combining the oligonucleotides which did not bind to the surfaces with no target molecules thereon with a cell culture containing the target molecule on their surface. Such a negative-positive selection protocol can be carried out in a medium containing human or bovine serum in order to select aptamers under simulated physiological conditions. It is desirable to replicate physiological conditions as closely as possible when carrying out the selection processes in that one endeavors to find oligonucleotides (aptamers) which bind to the target molecules under



physiological conditions so that such aptamers can later be used in vivo.

5 Aptamers which are selected in the presence of serum may be rendered nuclease-stable by the use of PCR primers with modified internucleotide linkages that are nuclease-stable as described in commonly assigned  
10 copending Application Publication No. WO90/15065 (incorporated herein by reference).

15 An alternative variation of the use of serum for aptamer selection is also possible. In accordance with this alternative protocol, candidate aptamers are added to a tissue culture medium lacking serum. The serum-free medium is incubated with cells which lack the target molecules on their surfaces. Following the  
20 incubation, a cell culture which contains the target molecules on their surfaces is combined with any oligonucleotides which did not bind to the first cell culture which did not have the target molecule thereon. This step in the protocol provides for positive  
25 selection. After continuing the incubation for the positive selection for 20-40 minutes, serum is added in order to provide a final concentration in the range of about 5% to 10% of serum. At this point, any  
30 oligonucleotides which are not tightly complexed with the target molecule begin to degrade due to the nucleases present in the added serum. However, the oligonucleotides which are tightly bound to the target molecules on the cells are nuclease resistant as they are inaccessible to the nucleases due to their physical  
35 association with the target molecules. After exposure to the nucleases for 10-30 minutes, the medium (i.e., the serum containing the nucleases) is removed and the cells are washed and caused to release the oligonucleotides or aptamers bound thereto by treatment of the cells with proteases and/or detergents. Any oligomers which are

substantially degraded by the nucleases will not be amplified during amplification processing.

In more detail, the present inventors have found that the nuclease activity present within the serum is primarily a 3' exonuclease activity. The presence of 3' exonuclease activity during target binding may be used with a candidate aptamer pool that has a short primer at the 3' end as a nuclease target. Accordingly, if the 3' end, which includes the primer, is degraded by the nuclease, the oligonucleotides attached to the degraded primers will not be amplified during amplification processing and will thereby be eliminated. A similar short primer sequence (6-10 bases) at the 5' end could also be utilized in the same manner if 5' exonucleases are added to the medium during the selection protocol.

At various stages of the screening process, advantage may be taken of PCR techniques for amplification of selected aptamer pools. While the material recovered after a single cycle of positive and negative selection may in some instances be suitable after amplification for sequencing directly, it is often advantageous to repeat the cycle until a lower dissociation constant ( $K_d$ ) is obtained for binding of the single-stranded oligonucleotide species to the transfectant cells (the first tissue culture cells) relative to the parental cells (the second tissue culture cells). Usually, multiple rounds of selection and aptamer amplification will be necessary in order to provide multiple opportunities to enrich for aptamers that specifically bind to the target structure. In addition, it is clearly within the scope of the present invention to amplify the selected pools of aptamer after each screening (positive or negative).

If an agonist or other substance already known to bind the desired target is available, competitive

binding analyses can be performed using the selected oligonucleotide species and radiolabeled substance. Depending upon the results of such competitive analyses, it can be determined whether it would be desirable to proceed with additional positive/negative screening cycles.

One could also determine whether the selected oligonucleotide species can inhibit the target protein in a functional assay. For example, oligonucleotides selected for binding to CD4, the human lymphocyte transmembrane protein, may be tested for their ability to inhibit HIV-1 infection of human lymphocytes in culture.

Modified Method Wherein Target/Aptamer Complexes are Separated from Solid Support

As set forth hereinabove, the original oligonucleotide mixture can be synthesized according to the desired contents of the mixture and can be separated by adding the oligonucleotide mixture to a column containing covalently attached target molecules (see, Ellington, A.D., et al., Nature (1990) 346:818-822) or to the target agents in solution (see Blackwell et al., Science (1990) 250:1104-1110; Blackwell et al., Science (1990) 250:1149-1151; or to the target agent bound to a filter (see Tuerk, C., and Gold, L., Science (1990) 249:505-510). Complexes between the aptamer and targeted agent are separated from uncomplexed aptamers using any suitable technique, depending on the method used for complexation. For example, if columns are used, non-binding species are simply washed from the column using an appropriate buffer. Specifically bound material can then be eluted.

If binding occurs in solution, the complexes can be separated from the uncomplexed oligonucleotides using, for example, the mobility shift in electrophoresis

technique (EMSA), described in Davis, R.L., et al., Cell (1990) 60:733. In this method, aptamer-target molecule complexes are run on a gel and aptamers removed from the region of the gel where the target molecule runs.

5 Unbound oligomers migrate outside these regions and are separated away. Finally, if complexes are formed on filters, unbound aptamers are eluted using standard techniques and the desired aptamer recovered from the filters.

10 In a preferred method, separation of the complexes involves detachment of target-aptamer complexes from column matrices as follows.

A column or other support matrix having covalently or noncovalently coupled target molecules is synthesized. Any standard coupling reagent or procedure may be utilized, depending on the nature of the support and the target molecule. For example, covalent binding may include the formation of disulfide, ether, ester or amide linkages. The length of the linkers used may be varied by conventional means. Noncovalent linkages include antibody-antigen interactions, protein-sugar interactions, as between, for example, a lectin column and a naturally-occurring oligosaccharide unit on a peptide.

25 Lectins are proteins or glycoproteins that can bind to complex carbohydrates or oligosaccharide units on glycoproteins, and are well-described in The Lectins (I.E. Liener et al., eds., Academic Press 1986). Lectins are isolated from a wide variety of natural sources, including peas, beans, lentils, pokeweed and snails. Concanavalin A is a particularly useful lectin.

30 Other linking chemistries are also available. For example, disulfide-derivatized biotin (Pierce) may be linked to a target molecule by coupling through an amine or other functional group. The resulting target-S-S-

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biotin complex could then be used in combination with  
avidin-derivatized support. Oligonucleotide-target  
complexes could then be recovered by disulfide bond  
cleavage. Alternatively, target may be coupled via a  
5 cis-diol linker, and oligonucleotide-target complexes may  
be recovered by mild oxidation of the vicinal diol bond  
using  $\text{NaIO}_4$  or other appropriate reagents. Linking  
chemistries will be selected on the basis of (i)  
conditions or reagents necessary for maintaining the  
10 structure or activity of the target molecule, and/or (ii)  
chemical groups or moieties on the target molecule  
available for linking to the support.

The oligomer mixture is added to and incubated  
with the support to permit oligonucleotide-target  
15 complexation. Complexes between the oligonucleotides and  
target molecule are separated from uncomplexed  
oligonucleotides by removing unbound oligomers from the  
support environment. For example, if columns are used,  
nonbinding species are simply washed from the column  
20 using an appropriate buffer.

Following removal of unbound oligomers, the  
target molecules are uncoupled from the support. The  
uncoupling procedure depends on the nature of the  
coupling, as described above. Targets bound through  
25 disulfide linkages, for example, may be removed by adding  
a sulfhydryl reagent such as dithiothreitol or  $\beta$ -  
mercaptoethanol. Targets bound to lectin supports may be  
removed by adding a complementary monosaccharide (e.g.,  
 $\alpha$ -methyl-mannoside, N-acetylglucosamine, glucose, N-  
30 acetylgalactosamine, galactose or other saccharides for  
concanavalin A). Oligonucleotides specifically bound to  
the target can then be recovered by standard denaturation  
techniques such as phenol extraction.

The method of elution of target-oligonucleotide  
35 complex from a support has superior unexpected properties

when compared with standard oligonucleotide elution techniques. This invention is not dependent on the mechanism by which these superior properties occur. However, without wishing to be limited by any one  
5 mechanism, the following explanation is offered as to how more efficient elution is obtained. Certain support effects result from the binding of oligonucleotides to the support, or the support in conjunction with oligonucleotide or target. Removing oligonucleotide-  
10 target complexes enables the recovery of oligonucleotides specific to target only, while eliminating oligonucleotides binding to the support, or the support in conjunction with oligonucleotide or target. At each cycle of selection, this method may give up to 1,000-  
15 fold enrichment for specifically binding species. Selection with targets remaining bound to support gives less enrichment per cycle, making it necessary to go through many more cycles in order to get a good aptamer population.

20

#### Aptamer Pools of Varying Length

Aptamers can also be selected in the above methods using a pool of oligonucleotides that vary in length as the starting material. Thus, several pools of  
25 oligonucleotides having random sequences are synthesized that vary in length from e.g. 50 to 60 bases for each pool and containing the same flanking primer-binding sequences. Equal molar amounts of each pool are mixed and the variable-length pool is then used to select for  
30 aptamers that bind to the desired target substance, as described above. This protocol selects for the optimal species for target binding from the starting pool and does not limit aptamers to those of a given length.

Alternatively, several pools of mixed length  
35 aptamers can be used in parallel in separate selections

and then combined and further selected to obtain the optimal binders from the size range initially used. For example, three pools, A, B and C, can be used. Pool A can consist of oligonucleotides having random sequences that vary in length from e.g. 30 to 40 bases; pool B can have sequences varying in length from e.g. 40 to 50 bases; and pool C can have sequences varying in length from 50 to 60 bases. It is to be understood that the lengths described above are for illustrative purposes only. After selection to obtain binders from A, B, and C, all aptamers are mixed together. A number of rounds of selection are done as described above to obtain the best binders from the initial species selected in the 30- to 60-base range. Note that with this technique, not all possible species in some of the pools are used for selection. If the number of sites available for binding are increased, i.e., if a column is used and the size of the column increased, more species can be included for selection. Furthermore, this method allows for the selection of oligomers from the initial starting pool that are of optimal length for binding the targeted agent.

#### Derivatization

Aptamers containing the specific binding sequences discerned through the method of the invention can also be derivatized in various ways. For example, if the aptamer is to be used for separation of the target substance, conventionally the oligonucleotide will be derivatized to a solid support to permit chromatographic separation. If the oligonucleotide is to be used to label cellular components or otherwise for attaching a detectable moiety to target, the oligonucleotide will be derivatized to include a radionuclide, a fluorescent molecule, a chromophore or the like. If the oligonucleo-

5        tide is to be used in specific binding assays, coupling  
to solid support or detectable label, and the like are  
also desirable. If it is to be used in therapy, the  
oligonucleotide may be derivatized to include ligands  
10        which permit easier transit of cellular barriers, toxic  
moieties which aid in the therapeutic effect, or  
enzymatic activities which perform desired functions at  
the targeted site. The aptamer may also be included in a  
suitable expression system to provide for in situ  
10        generation of the desired sequence.

#### Consensus Sequences

15        When a number of individual, distinct aptamer  
sequences for a single target molecule have been obtained  
and sequenced as described above, the sequences may be  
examined for "consensus sequences." As used herein,  
"consensus sequence" refers to a nucleotide sequence or  
region (which may or may not be made up of contiguous  
nucleotides), which is found in one or more regions of at  
20        least two aptamers, the presence of which may be  
correlated with aptamer-to-target-binding or with aptamer  
structure.

25        A consensus sequence may be as short as three  
nucleotides long. It also may be made up of one or more  
noncontiguous sequences with nucleotide sequences or  
polymers of hundreds of bases long interspersed between  
the consensus sequences. Consensus sequences may be  
identified by sequence comparisons between individual  
aptamer species, which comparisons may be aided by  
30        computer programs and other tools for modeling secondary  
and tertiary structure from sequence information.  
Generally, the consensus sequence will contain at least  
about 3 to 20 nucleotides, more commonly from 6 to 10  
nucleotides.



-55-

As used herein "consensus sequence" means that certain positions, not necessarily contiguous, of an oligonucleotide are specified. By specified is meant that the composition of the position is other than completely random. Not all oligonucleotides in a mixture may have the same nucleotide at such position; for example, the consensus sequence may contain a known ratio of particular nucleotides. For example, a consensus sequence might consist of a series of four positions wherein the first position in all members of the mixture is A, the second position is 25% A, 35% T and 40% C, the third position is T in all oligonucleotides, and the fourth position is G in 50% of the oligonucleotides and C in 50% of the oligonucleotides.

When a consensus sequence is identified, oligonucleotides that contain that sequence may be made by conventional synthetic or recombinant means. These aptamers, termed "secondary aptamers," may also function as target-specific aptamers of this invention. A secondary aptamer may conserve the entire nucleotide sequence of an isolated aptamer, or may contain one or more additions, deletions or substitutions in the nucleotide sequence, as long as a consensus sequence is conserved. A mixture of secondary aptamers may also function as target-specific aptamers, wherein the mixture is a set of aptamers with a portion or portions of their nucleotide sequence being random or varying, and a conserved region which contains the consensus sequence. Additionally, secondary aptamers may be synthesized using one or more of the modified bases, sugars and linkages described herein using conventional techniques and those described herein.

35

Immune Recruitment

The present invention also provides a method whereby immune response is elicited in a desired manner through the use of agents which are directed to specific targets on cells involved in a pathological condition of interest. The aptamers prepared herein are useful as targeting agents in this method. In a particular embodiment of the invention, the known ability of various materials to elicit strong immune responses is exploited so as, in turn, to stimulate the immune response to target pathologic cells, which may themselves otherwise have the ability to reduce or escape effective CTL responses.

Pursuant to this method of the invention, in a first step a targeting agent is identified that specifically binds to a surface feature of the pathologic cells of interest. Once such a selective targeting agent has been identified, in a second step a conjugate is formed with a moiety known to act itself as an immunogen, for example as an antigen for eliciting a strong CTL response in the organism. By virtue of the selective binding of the targeting agent component of the conjugate to cells containing the target, these cells are in effect modified so as to exhibit the immunologic character of the associated immunogenic component of the conjugate. Thus, when the associated moiety is an antigen which elicits a strong CTL response, the cells are effectively marked for destruction by the antigen component of the conjugate.

In accordance with one preferred embodiment of the invention, the targeting agent is an oligonucleotide which binds to a specific target on a cell surface, and the immunomodulatory component of the conjugate is a polypeptide which elicits a strong CTL response.

Utility of the Aptamers

The aptamers of the invention are useful in diagnostic, research and therapeutic contexts. For diagnostic applications, aptamers are particularly well suited for binding to biomolecules that are identical or similar between different species. Classes of molecules such as kinins and eicosanoids generally do not serve as good antigens because they are not easily recognized as foreign by the immune systems of animals that can be used to generate antibodies. Antibodies are generally used to bind analytes that are detected or quantitated in various diagnostic assays. Aptamers represent a class of molecules that may be used in place of antibodies for diagnostic and purification purposes.

The aptamers of the invention are therefore particularly useful as diagnostic reagents to detect the presence or absence of the target substances to which they specifically bind. Such diagnostic tests are conducted by contacting a sample with the specifically binding oligonucleotide to obtain a complex which is then detected by conventional means. For example, the aptamers may be labeled using radioactive, fluorescent, or chromogenic labels and the presence of label bound to solid support to which the target substance has been bound through a specific or nonspecific binding means detected. Alternatively, the specifically binding aptamers may be used to effect initial complexation to the support. Means for conducting assays using such oligomers as specific binding partners are generally known to track those for standard specific binding partner based assays.

This invention also permits the recovery and deduction of oligomeric sequences which bind specifically to cell surface proteins and specific portions thereof. Therefore, these oligonucleotides can be used as a

separation tool for retrieving the substances to which they specifically bind. By coupling the oligonucleotides containing the specifically binding sequences to a solid support, for example, proteins or other cellular components to which they bind can be recovered in useful quantities. In addition, these oligonucleotides can be used in diagnosis by employing them in specific binding assays for the target substances. When suitably labeled using detectable moieties such as radioisotopes, the specifically binding oligonucleotides can also be used for in vivo imaging or histological analysis.

It may be commented that the mechanism by which the specifically binding oligomers of the invention interfere with or inhibit the activity of a target substance is not always established, and is not a part of the invention. The oligomers of the invention are characterized by their ability to target specific substances regardless of the mechanisms of targeting or the mechanism of the effect thereof.

For use in research, the specifically binding oligonucleotides of the invention are especially helpful in effecting the isolation and purification of substances to which they bind. For this application, typically, the oligonucleotide containing the specific binding sequences is conjugated to a solid support and used as an affinity ligand in chromatographic separation of the target substance. The affinity ligand can also be used to recover previously unknown substances from sources which do not contain the target substance by virtue of binding similarity between the intended target and the unknown substances. Furthermore, as data accumulate with respect to the nature of the nonoligonucleotide/oligonucleotide-specific binding, insight may be gained as to the mechanisms for control of gene expression.

In therapeutic applications, the aptamers of the invention can be formulated for a variety of modes of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, latest edition.

For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the aptamers of the invention are formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the aptamers may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

Systemic administration can also be by transmucosal or transdermal means, or the oligomers can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays, for example, or using suppositories. For oral administration, the oligomers are formulated into conventional oral administration forms such as capsules, tablets, and tonics.

For topical administration, the oligomers of the invention are formulated into ointments, salves, gels, or creams, as is generally known in the art.

The oligonucleotides may also be employed in expression systems, which are administered according to

techniques applicable, for instance, in applying gene therapy.

#### Immune Response Modulation

5           The present invention is also directed to a method whereby immune response is elicited in a desired manner through the use of agents which are directed to specific targets on cells involved in a pathological condition of interest.

10

#### Targeting Agents

          For use as targeting agents, any of a number of different materials which bind to cell surface antigens may be employed. When available, antibodies to target  
15   cell surface antigens will generally exhibit the necessary specificity for the target. Similarly, ligands for any receptors on the surface of the pathologic cells of interest may suitably be employed as targeting agent. Yet another class of potentially valuable targeting  
20   agents is oligonucleotides of the requisite binding selectivity.

          Typically, for reaction with antigenic determinants on proteins, antibodies raised against these proteins, either polyclonal or monoclonal, may be used.  
25   Polyclonal anti-sera are prepared in conventional ways, for example by injecting a suitable mammal with antigen to which antibody is desired, assaying the antibody level in serum against the antigen, and preparing anti-sera when the titers are high. Monoclonal antibody  
30   preparations may also be prepared conventionally, such as by the method of Koehler and Milstein using, e.g., peripheral blood lymphocytes or spleen cells from immunized animals and immortalizing these cells either by viral infection, by fusion with myelomas, or by other

35

conventional procedures and screening for the production of the desired antibodies by isolated colonies.

In addition to antibodies, suitable immunoreactive fragments may also be employed, such as the Fab, Fab', or F(ab')<sub>2</sub> fragments. Many antibodies suitable for use in forming the targeting mechanism are already available in the art. For example, the use of immunologically reactive fragments as substitutes for whole antibodies is described by Spiegelberg, H.L., in "Immunoassays in the Clinical Laboratory" (1978) 3:1-23.

One known surface antigen to which antibodies can be raised, for example, is the extracellular domain of the HER2/nu associated with breast tumors. As set forth by Fendly, B.M. et al. J Biol Resp Mod (1990) 2:449-455, antibodies to HER/nu can be raised in alternate hosts (since the antigen is not foreign to the host-bearing tumor) and can be used in immunoconjugates to bind specifically to the tumor. As applied in the method of the invention, the antibody or fragment thereof would be coupled not to a toxin, but to an immunomodulatory agent which would mount a CTL response.

In addition to immunoreactivity, targeting can be effected by utilizing receptor ligands which target receptors at the target cell surface, for example on the basis of complementarity of contours or charge patterns between the receptor and ligand. As used herein, the term "receptor ligand" refers to any substance, natural or synthetic, which binds specifically to a cell surface receptor, protein or glycoprotein found at the surface of the desired target cell population. These receptor ligands include lymphokine factors, for example, IL2 or viral or tumor antigens.

Oligonucleotides identified as binding to one or more surface antigen of the pathologic cells may also be used to form conjugates in a known manner, and are

particularly preferred for use as targeting agents in accordance with the present invention.

#### Immunomodulatory Agents

5           An "immunological response" as discussed herein generally refers to the development in a mammal of either a cell- or antibody-mediated immune response to an agent of interest. Typically, such a response consists of the mammal producing antibodies and/or cytotoxic T-cells  
10           directed specifically to a particular agent. In the context of the present invention, however, an "immunological response different from that elicited by the pathologic cell itself in the absence of the conjugate" may constitute, e.g., the failure to produce  
15           antibodies or cytotoxic T-cells under circumstances (for example, in the presence of a particular antigen) which would normally result in the induction of a specific response.

          Examples of moieties known to act as antigens  
20           for eliciting a strong CTL response include a wide range of biologically active materials. Particularly suitable for use in this regard are short peptide sequences, such as those which may correspond to the antigenic determinants of known immunogenic proteins. For example,  
25           sequences derived from viral or bacterial pathogens may be useful in stimulating a strong CTL response in the infected host organism.

          Other immunomodulatory agents useful in the invention include fragments of the HLA Class I  
30           glycoproteins. The ability of such HLA Class I glycoproteins or fragments thereof to stimulate a CTL response has been documented by Symington, F.W. et al., J Invest Dermatol (1990) 95:224-228. Also known to elicit CTL responses are short regions of viral antigens such as  
35           those of the influenza virus nucleoprotein (Rothbard,



J.B. et al., EMBO J (1989) 8:2321-2328) and sections of the murine minor histocompatibility antigens such as H-25.3 cell surface antigen (Lai, P.K., Transplantation (1985) 39:638-643). Other known agents which expand CTL as opposed to helper T-cells include interleukin-6 and cyclosporin A.

#### Techniques for Coupling Targeting Agents and Immunomodulatory Agents

10                   Coupling of the targeting agents with the immunomodulatory agents may be carried out using any of a variety of different techniques which are well known per se to those working in the field. The particular choice of coupling method depends on the chemical nature of the specific targeting and immunomodulatory agents.

15                   Selection of the most appropriate method of coupling from among the variety of available alternatives for any given types of targeting and immunomodulatory agents may in some instances require routine screening to determine empirically which conjugates provide the optimum combination of targeting specificity and desired immunomodulatory effect.

25                   When at least one of the agents which constitute the conjugate is a polypeptide, well-known chemical methods for formation of chemical bonds with, e.g., functional groups on amino acid side-chains or preferably N-terminal amino or C-terminal carboxyl groups, may be employed. One common approach is the use of linkers which may be homobifunctional or

30                   heterobifunctional, and typically involve highly reactive functional groups on the linker. Another approach is the use of dehydrating agents, such as carbodiimides, to effect the formation of new bonds by reaction of a carboxyl moiety on one member of the conjugate with a

35                   free amino group on the other. Particularly suitable

methods involving the use of conjugation reagents (i.e., reagents which result in elimination of water to form a new covalent bond) are discussed in, e.g., U.S. Patent 4,843,147 to Levy et al., which is hereby incorporated by reference. Additional techniques for formation of conjugates between polypeptides and various types of biologically active molecules have been described in the art with respect to the formation of cytotoxic conjugates; for example, a variety of different conjugate-forming reactions are described in U.S. Patent 4,507,234 to Kato et al., which is also hereby incorporated by reference.

Similarly, methods are known for attaching a variety of different species to oligonucleotides. For example, Asseline, U. et al. (Proc Natl Acad Sci 81, 3297-3301 (1984)) describes the covalent linking of an intercalating agent via a polymethylene linker through a 3'-phosphate group. Mori, K. et al. (FEBS Letters 249:213-218 (1989)) describes the covalent attachment of groups via a methylene linker at the 5'-terminus of oligonucleotides. PCT application WO89/05853 published June 29, 1989, the entire disclosure of which is hereby incorporated by reference, describes a variety of methods for formation of conjugates between nucleotide sequences and chelating agents; the chelating agent is joined to the nucleotides sequence by either a covalent bond or a linking unit derived from a polyvalent functional group. Other methods will of course be readily apparent to those working in the field.

#### Identification of Suitable Targets for the Conjugates

Suitable targets for binding a targeting agent include cell surface antigens which are specific to the pathologic cells which it is desired to treat. For example, most tumor antigens (such as the

carcinoembryonic antigen associated with several types of cancer) do not generally elicit an effective CTL response. The presence of the antigens on the surface of tumor cells enables the use of appropriately tailored targeting agents to deliver conjugate specifically to those cells.

In addition to eliciting CTL type responses, other types of immunomodulatory effects may be achieved through the use of the inventive conjugates. For example, the conjugates of the invention may be useful in preventing the progression or the cure of autoimmune disease.

Diseases with an autoimmune component, such as diabetes or arthritis, appear to involve response to specific self antigens. By using conjugates of the invention to elicit an immune response against those immune cells which mediate the attack on self tissues, a positive effect on the course of the disease may be achieved. In principle, a group of antigenically related immune cells that mediate the systemically inappropriate response could be targeted using a single conjugate specific for that antigen. Destruction of this marked population by the immune system should lead to an amelioration of the disease condition.

Alternatively, the binding of appropriate conjugates to target antigens on cells could be employed as a means to mask recognition of those antigens or of the cell bearing the antigens. This could prevent the destruction of the cell carrying the antigens, and thus result in stasis of autoimmune disease progression.

Further, an immune response stimulated by the immunomodulatory portion of the conjugate may result in other desirable immunologic responses in the organism. In particular, by virtue of the cell death process initiated in response to the conjugate, a highly

desirable response to other unmarked cells of the same type may result. Thus, by identifying a particular class of cells for recognition by various components of the immune system via the immunomodulatory portion of the conjugate, it may be possible to induce a particular category of response (e.g., CTL-mediated destruction) to that category of cells as a whole, regardless of whether or not the cells are marked by conjugate.

Target molecules that are not conventionally considered to be biomolecules are also appropriate for the methods described herein. Examples of "non-biomolecule" targets include intermediates or end-products generated by chemical synthesis of compounds used in therapeutic, manufacturing or cosmetic applications, including polymer surfaces, especially those useful in medical applications. Aptamer oligonucleotides may be used to specifically bind to most organic compounds and are suitably used for isolation or detection of such compounds.

The following examples are meant to illustrate, but not to limit the invention.

#### Example 1

##### Selection of Aptamers that Bind to Bradykinin

25

##### A. Preparation of Bradykinin Column

Bradykinin derivatized Toyopearl™ (Toso Haas, Inc., Woburn, MA) support was used for all selections described. Bradykinin was coupled to the Toyopearl support through its amino termini according to the manufacturer's instructions. Bradykinin (NH<sub>2</sub>-arg-pro-pro-gly-phe-ser-pro-phe-arg-COOH, acetate salt) was obtained from Bachem Feinchemikalien AG (Cat. No. H-1970). Toyopearl AF-carboxyl 650 M was converted to the NHS-ester by treatment with N-hydroxy succinimide (NHS)

and diisopropyl carbodiimide in dioxane/DMF (1:1) for 24 hours. The support was washed with DMF, H<sub>2</sub>O, 200 mM NaHCO<sub>3</sub> and treated with a solution of bradykinin (20 mg of bradykinin/ml support) in 200 mM NaHCO<sub>3</sub> for 3 days.

5 The support was then washed and the coupling yield was determined by HCl digestion of the support (80°C for 8 hours) and a ninhydrin assay using free bradykinin as a standard. The yield was found to be 16 mg/ml support (16 μmole/ml support). The coupled support was then capped  
10 by treatment with acetic acid (NHS-ester) in dioxane/200 mM NaHCO<sub>3</sub> buffer (1:1).

An underivatized capped support to be used as a control was made by treating Toyopearl AF-carboxyl 650M with acetic acid (NHS-ester) in dioxane/200 mM NaHCO<sub>3</sub>  
15 buffer (1:1), followed by washing.

#### B. Synthesis of Oligonucleotide Pool

DNA oligonucleotides containing a randomized sequence region were synthesized using standard solid  
20 phase techniques and phosphoramidite chemistry (Oligonucleotide Synthesis, Gait, M.J., ed. (IRL Press), 1984; Cocuzza, A., Tetrahedron Letters, (1989) 30:6287-6291.) A 1 μM small-scale synthesis yielded 60 nmole of HPLC-purified single-stranded randomized DNA. Each  
25 strand consisted of specific 18-mer sequences at both the 5' and 3' ends of the strand and a random 60-mer sequence in the center of the oligomer to generate a pool of 96-mers with the following sequence (N = G, A, T or C):

30 5' HO-CGTACGGTCGACGCTAGCN<sub>60</sub>CACGTGGAGCTCGGATCC-OH 3'

DNA 18-mers with the following sequences were used as primers for PCR amplification of oligonucleotide sequences recovered from bradykinin columns. The 5'  
35 primer sequence was 5' HO-CGTACGGTCGACGCTAGC-OH 3' and

the 3' primer sequence was 5' biotin-O-GGATCCGAGCTCCACGTG-OH 3'. The biotin residue was linked to the 5' end of the 3' primer using commercially available biotin phosphoramidite (New England Nuclear, Cat. No. NEF-707). The biotin phosphoramidite is incorporated into the strand during solid phase DNA synthesis using standard synthesis conditions.

10 C. Selection for Aptamers That Bind to an Immobilized  
Bradykinin Column

400  $\mu$ l bradykinin-derivatized Toyopearl support was loaded on a 1.5 ml column housing. The column was washed with 3 ml of 20mM Tris-acetate buffer (pH 7.4) containing 1mM  $MgCl_2$ , 1 mM  $CaCl_2$ , 5mM KCl and 140mM NaCl (the "selection buffer"). An identical column was prepared using the underivatized Toyopearl control support described in Example 1-A.

20 An initial oligonucleotide pool (0.5 nmole,  $3 \times 10^{14}$  unique sequences) of synthetic 96-mers prepared in Example 2 was amplified approximately 30-fold by large-scale PCR using known techniques. Assuming 10-20% readthrough of synthetic DNA and possible preferential amplification by the Taq polymerase, the estimated actual complexity was reduced to about  $1 \times 10^{13}$  unique sequences.

25 This amplified oligonucleotide pool (0.1 nmoles, about 6 copies of  $1 \times 10^{13}$  unique sequences), doped with 5'- $^{32}P$ -labeled species, was used in the first selection round. The pool was heated to 94°C for 3 minutes in selection buffer, allowed to cool to room temperature, applied to the control column in a volume of 100  $\mu$ l, and allowed to equilibrate for approximately 10 minutes. The column was then eluted with selection buffer and the eluent collected in 200  $\mu$ l fractions. The bulk of the counts (approximately 95%) with little

35

affinity for the matrix eluted in the first 2 or 3 fractions after the void volume. These fractions were combined, applied to the bradykinin-linked support (400  $\mu$ l support, approximately 5  $\mu$ mole, washed with 3 ml of selection buffer), and eluted with selection buffer. The column was then eluted with selection buffer and the eluent collected in 200  $\mu$ l fractions. Fractions were collected until the eluted counts in a fraction plateaued at less than about .05% total loaded counts. The column was then eluted with elution buffer (500 mM Tris·HCl (pH 8.3), 20 mM EDTA) at room temperature. Aptamers were eluted in the first 2 or 3 fractions after the void volume. These fractions were combined and precipitated using ethanol and glycogen as the carrier. The aptamer pellet was resuspended in 200  $\mu$ l of ddH<sub>2</sub>O (deionized distilled water) and divided into two 0.5 ml siliconized Eppendorf tubes for PCR. All remaining counts on the column were removed by treatment with 0.1N NaOH (0.5 ml), although these species were not used in subsequent amplification and selections.

#### D. Amplification of Selected Aptamers

Two groups of selected aptamers were amplified by PCR using standard techniques and the following protocol.

A 200  $\mu$ l PCR reaction consisted of the following: 100  $\mu$ l template aptamer (approximately 2 pmoles); 20  $\mu$ l buffer (100 mM Tris·Cl (pH 8.3), 500 mM KCl, 20 mM MgCl<sub>2</sub>); 32  $\mu$ l NTP's (5 mM conc total, 1.25 mM each ATP, CTP, GTP, and TTP); 20  $\mu$ l primer 1 (biotinylated 18-mer, 50  $\mu$ M); 20  $\mu$ l primer 2 (18-mer, 50  $\mu$ M); 2  $\mu$ l hot NTP's (approximately 2 mCi); 6  $\mu$ l ddH<sub>2</sub>O; and 2  $\mu$ l Taq I Polymerase (10 units). The reaction was sealed with 2 drops NUJOL mineral oil. A control reaction was also performed without template aptamer.

Initial denaturation was at 94°C for 3 minutes, but subsequent denaturation after each elongation reaction lasted 1 minute. Primer annealing occurred at 60°C for 1 minute, and elongation of primed DNA strands using the Taq polymerase ran at 72°C for 2 minutes. The final elongation reaction to completely fill in all strands ran for 10 minutes at 72°C, and the reaction was then held at 4°C.

Fifteen rounds of Taq polymerase elongation were carried out in order to amplify the selected aptamer DNA. After the reactions were completed, the NUJOL oil was removed by chloroform extraction. The two reactions were combined and chloroform extracted again. A 2 µl sample was removed from each of the aptamer and control reaction for counting and an analytical gel. The rest of the amplified aptamer was run over four Nick columns (G-50 Sephadex, washed with 3 ml TE buffer (10 mM Tris-HCl (pH 7.6), 0.1 mM EDTA)) to remove unincorporated NTP's, primers, and salt. 100 µl of the amplified aptamer pool (400 µl total) was applied to each Nick column. 400 µl of TE buffer was then added to each column and the columns were eluted with an additional 400 µl using 10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA (1600 µl total). A 8 µl sample was removed from the combined eluents for counting and an analytical gel. The remaining eluent was loaded on an avidin agarose column (Vector Laboratories, Cat. No. A-2010) (600 µl settled support, washed with 3 x 800 µl TE buffer). Approximately 90% of the loaded counts remained on the column. The column was washed with TE buffer (3 x 800 µl) and then the nonbiotinylated strand was eluted with 0.15 N NaOH (400 µl fractions). More than 45% of the counts on the column were eluted in the first two fractions. These two fractions (800 µl) were combined and neutralized with approximately 4 µl of glacial acetic acid. The neutralized fractions were



reduced to 200  $\mu$ l by speed vacuum or butanol extraction and then precipitated with EtOH. The resultant pellet was dissolved in 102  $\mu$ l selection buffer, heated at 94°C for 3 min, and cooled to room temperature. A 2  $\mu$ l sample  
5 was removed for counting and an analytical gel.

E. Aptamer Recovery Profiles From the First Two Rounds of Selection

Aptamers eluted from the first round of  
10 bradykinin-linked column selection were obtained in two 100  $\mu$ l fractions that contained 0.07% of the total counts loaded. Recovery of three 100  $\mu$ l fractions from the second round selection yielded 0.26% of the total counts loaded therein, indicating that an increased proportion  
15 of the aptamers loaded onto the column had bound to bradykinin.

F. Further Rounds of Aptamer Selection on Bradykinin Columns

20 Additional rounds of selection and amplification were carried out in order to obtain a population of aptamers that consisted of species that bound to bradykinin. The cycle of Examples 1-C and 1-D was repeated 6 times until a significant portion of the  
25 oligonucleotide pool (as measured by cpm) remained on the column after washing with selection buffer. Under the selection and amplification conditions used, about 15% of input counts (0.5 nmole DNA, about 19  $\mu$ g) bound to the bradykinin column in rounds 5 and 6. About 6% of the  
30 counts bound to the control column. However, the proportion of counts that bound to the bradykinin column was higher, 40% of input cpm, when the initial amount of input DNA was reduced from 0.5 nmole to 0.1 nmole. Under these conditions (0.1 nmole input DNA, about 3.5  $\mu$ g) 19%  
35 of the counts bound to the capped control column. The

relatively high proportion of counts bound to the control column was due to overloading of the control column during the prebinding process prior to adding aptamer to bradykinin columns at each round of selection. This high level of binding to the control column in the later round pools (rounds 5 and 6) can be reduced by reducing the molar ratio of input DNA to column during the selection process. This protocol is described in Example 1-G below. This high affinity aptamer pool was eluted, amplified by PCR, cloned, and sequenced (about 20 to 40 clones). From these clones, several homologous batches of aptamers and/or individual clones are prepared by solid phase DNA synthesis and tested for bradykinin binding affinity and specificity.

G. Aptamer Selection Using a Reduced Molar Ratio of Aptamer to Column

An initial oligonucleotide pool (0.5 nmole,  $3 \times 10^{14}$  unique sequences) of synthetic 96-mer prepared as in Example 1-B is amplified approximately 30-fold by large-scale PCR using known techniques. Assuming 10-20% readthrough of synthetic DNA and possible preferential amplification by the Taq polymerase, the estimated actual complexity is reduced to about  $1 \times 10^{13}$  unique sequences.

This amplified oligonucleotide pool (0.5 nmoles, about 30 copies of  $1 \times 10^{13}$  unique sequences) doped with 5'- $^{32}\text{P}$ -labeled species, is used in the first selection round. A bradykinin-linked column and control support column are prepared as in Example 1-B.

The pool is heated to 94°C for 3 minutes in selection buffer, cooled to room temperature, then applied to 1 ml of control support washed with 3 ml of selection buffer, and allowed to equilibrate for about 10 minutes. The column is then eluted with selection buffer and the eluent collected in 200  $\mu\text{l}$  fractions. The bulk

of the counts (approximately 90%), with little affinity for the matrix is eluted in the first 2 or 3 fractions after the void volume. These fractions are combined, applied to the bradykinin-linked support (1 ml, approximately 10 to 15  $\mu$ mole, washed with 3 ml of selection buffer), eluted with selection buffer, and the eluent collected in 200  $\mu$ l fractions. Fractions are collected until the eluted counts in a fraction plateau at less than 0.05% of the total counts loaded on the column (approximately 12 fractions). The column is then eluted with elution buffer (.15 N NaOH, 50 mM EDTA). The aptamers are eluted in the first 2 or 3 fractions after the void volume. These fractions are combined and precipitated using ethanol and glycogen as the carrier. The aptamer pellet is taken up in 100  $\mu$ l of dd H<sub>2</sub>O and transferred to a 0.5 ml siliconized eppendorf tube for PCR. One aptamer PCR reaction and one control (without template) reaction are then run as described in Example 1-D.

The above procedure is then repeated, with the exception that the oligonucleotide pool used in subsequent selection cycles is reduced to 0.1 nmole and the control and bradykinin support volumes are reduced to about 330  $\mu$ l (about 3 to 5  $\mu$ moles bradykinin). The procedure is repeated (~5-6 times) until a significant portion of oligonucleotide remains on the column after washing with selection buffer. This high-affinity aptamer pool is eluted, converted to double stranded DNA by PCR, and cloned. About 20 clones are sequenced. From these clones, several homologous batches of aptamers are prepared and tested for binding affinity and target specificity. High affinity aptamers are mutagenized using the techniques described in Ellington et al., Nature (1990) 346:818-822 to yield a 15% mutation rate at

each position and reselected to determine those bases which are involved in binding.

### Example 2

#### 5      Selection of Aptamers that Bind to PGF2 $\alpha$

##### A. Preparation of PGF2 $\alpha$ Linked to a Solid Support

PGF2 $\alpha$  derivatized Toyopearl™ AF-amino 650M (Toso Haas, Inc., Woburn, MA) support (charged with 10  
10       $\mu$ moles PGF2 $\alpha$ /ml matrix) was used for all selections described. The support was coupled through the free carboxyl group of PGF2 $\alpha$  according to the manufacturer's instructions. PGF2 $\alpha$  was purchased from Sigma Chemical Co. (Cat. No. P 3023) and tritiated PGF2 $\alpha$  was purchased  
15      from New England Nuclear.

10 mg of PGF2 $\alpha$  (Tris salt) was dissolved in 1 ml H<sub>2</sub>O/methanol and converted to the sodium salt by passage over an ion exchange column. The column eluent was then evaporated, dissolved in dioxane and converted  
20      to the N-hydroxy-succinimide (NHS) ester by treatment with NHS and diisopropyl carbodiimide for 24 hrs. This mixture was then added to 1 ml of the settled support Toyopearl washed previously with 200 mM NaHCO<sub>3</sub>. The mixture was shaken for 24 hrs., and washed with a NaHCO<sub>3</sub>  
25      solution. To determine the amount of coupling, the above described procedure was repeated except that a small amount of tritiated PGF2 $\alpha$  was added. The coupling yield was determined by the amount of tritium associated with the support. The support was then capped by treatment  
30      with acetic acid (NHS-ester) in dioxane/200 mM NaHCO<sub>3</sub> buffer (1:1).

An underivatized capped support was made by treating Toyopearl AF-amino 650M with acetic acid (NHS-ester) in dioxane/200 mM NaHCO<sub>3</sub> buffer (1:1) to be used  
35      as a control.

B. Selection for Aptamers That Bind to an Immobilized  
PGF2 $\alpha$  Column

200  $\mu$ l derivatized Toyopearl support containing  
5 2  $\mu$ mole of PGF2 $\alpha$  ligand was loaded on a 1.5 ml column  
housing. The column was washed with 3 ml of 20mM Tris-  
acetate buffer (pH 7.4) containing 1mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>,  
5mM KCl and 140mM NaCl (the "selection buffer"). An  
identical column was prepared using the underivatized  
10 Toyopearl control support described in Example 2-A.

0.5 nmoles of the oligonucleotide pool prepared  
in Example 1-B (doped with tracer amounts of 5'-<sup>32</sup>P-end-  
labeled species) was resuspended in 400  $\mu$ l of selection  
buffer and heat denatured for 2 min at 95°C. The  
15 denatured DNA was immediately transferred to wet ice for  
10 min. This material was applied to the control support  
(underivatized Toyopearl), flow initiated, and eluent  
collected. Flow-through was reapplied three times. At  
the end of the third application, the column was rinsed  
20 with 200  $\mu$ l selection buffer (1 bed volume). The flow-  
through was pooled and applied for a fourth time. A  
column profile was established using <sup>32</sup>P quantification  
via Cerenkov counting. Flow-through material was then  
pooled for application to the PGF2 $\alpha$  support.

25 Application of the flow-through pool to PGF2 $\alpha$ -  
derivatized Toyopearl was performed as described above.  
After the third application, the column was washed with  
200  $\mu$ l of selection buffer and the material reapplied to  
establish a column profile. The support was washed with  
30 additional selection buffer until the eluting <sup>32</sup>P  
material decreased to low levels, less than 0.2% of  
initial input cpm. The support was then washed with 1ml  
of selection buffer containing 1M NaCl. Bound  
oligonucleotides were eluted with 20mM EDTA/60%  
35 acetonitrile. The solvent was removed under vacuum and

the material chromatographed on a Nick column (Pharmacia, G-50 Sephadex columns) as per the manufacturer's instructions using 10mM Tris (pH 7.5)/0.1mM EDTA/250mM NaCl. The  $^{32}\text{P}$ -containing fraction was then precipitated  
5 with 20 $\mu\text{g}$  of carrier glycogen and absolute ethanol (2.5 vol) on dry ice for 15 minutes. The DNA was pelleted for 15 minutes at 4°C, washed with 70% ethanol, and dried under vacuum.

10 C. Amplification of Aptamers Obtained After Selection on a PFG2a Column

The DNA selected in Example 2-B above was amplified via PCR using known techniques under the following conditions: 1 nmole of 5' and 3' primer  
15 (biotinylated), 250  $\mu\text{M}$  dNTPs (containing 20  $\mu\text{Ci}$  each of dCTP, dGTP and dATP) in 200  $\mu\text{l}$  of 10 mM Tris (pH 8.3) containing 50 mM KCl and 1.5 mM  $\text{MgCl}_2$ . The reaction vessel was sealed with mineral oil, and subjected to 25  
20 cycles of amplification. The mineral oil was then removed, and 100  $\mu\text{l}$   $\text{CHCl}_3$  was added. The solution was then vortexed and separated via centrifugation. The aqueous layer was removed, concentrated via n-butanol extraction and brought to a final volume of 100  $\mu\text{l}$ . The  
25  $^{32}\text{P}$  labeled DNA was then passed over a Nick column equilibrated in 100 mM Tris (pH 7.5)/100 mM NaCl to remove unincorporated primer and dNTPs. The column eluent was then applied to 400  $\mu\text{l}$  of avidin-agarose matrix (two applications resulted in more than 90%  
30 retention of the input). The matrix was extensively washed to remove contaminants and single-stranded aptamer eluted with 600  $\mu\text{l}$  washes of 0.15N NaOH (2X), yielding 40-48% recovery of input  $^{32}\text{P}$  DNA. The aptamer solution was brought to pH 6 with acetic acid and concentrated via  
35 n-butanol extraction to 40% of the initial volume. The material was precipitated with absolute ethanol (3 vols)

-77-

on dry ice for 15 minutes. The DNA was pelleted, washed with 70% ethanol and dried under vacuum. The material was resuspended in selection buffer as described above. Subsequent rounds of selection were carried out using the same protocol: removal of aptamer by binding to the control support column; followed by binding to the PGF2 $\alpha$  column. Each round of selection resulted in a pool enriched in the aptamer that specifically bound to the PGF2 $\alpha$  immobilized on the column. Amplified material was always obtained from the PGF2 $\alpha$  column by elution in 20 mM EDTA/60% acetonitrile.

D. Quantitation of Aptamer Recovery From PGF2 $\alpha$  Columns After 6 Rounds of Selection

The total radioactivity ( $^{32}\text{P}$ ) associated with each oligonucleotide pool used for PGF2 $\alpha$  selection was determined prior to addition to underivatized Toyopearl columns. DNA from underivatized and PGF2 $\alpha$ -derivatized columns was recovered and total radioactivity determined and expressed as % recovery. Data for  $^{32}\text{P}$  recovered (in cpm) after column washes are shown in Table 2 for selection rounds 1 through 6.

Table 2

	<u>Round of selection</u>	<u>% Total cpm Eluted by CH<sub>3</sub>CN/EDTA Wash</u>
5	1	0.37
	2	2.31
	3	7.98
	4	16.97
	5*	17.34
10	6	17

\*total cpm recovered after 2 column washes with CH<sub>3</sub>CN/EDTA.

#### E. Characterization of Aptamers Eluted From the

##### 15 Round 6 Column

(a) The recovery of specifically-binding oligonucleotides in each amplified pool from round 4, 5 and 6 selections remained constant at about 17% of total input cpm. Aptamers obtained from the round 6 column washes prior to addition of CH<sub>3</sub>CN/EDTA were recovered by ethanol precipitation, pooled, and subjected to selection on a new PGF2 $\alpha$  column. The total cpm recovered from CH<sub>3</sub>CN/EDTA elution was about 17%.

This demonstrates that the aptamers eluted by CH<sub>3</sub>CN/EDTA in round 6 specifically bind to the PGF2 $\alpha$  ligand. The 17% recovery was due only to the limited binding capacity of the PGF2 $\alpha$  column. This means that 1 to 10% of linked PGF2 $\alpha$  is available for aptamer binding, giving a ligand:oligonucleotide loading ratio of about 40 to 400. Higher recovery values for round 4 through 6 selections have been reported but result from a higher ligand:oligonucleotide ratio of about 10-30,000 (Ellington and Szostak, Nature (1990) 346:818-822). Thus, the aptamers obtained after 6 rounds of PGF2 $\alpha$  selection (the "round 6 pool") were a pool of molecules



that resulted from competition among aptamer species for a limited number of PGF2 $\alpha$  binding sites.

(b) The round 6 pool was further characterized by adding 1 ml of a 2.4 mg/ml solution (5  $\mu$ mole) of PGF2 $\alpha$  in selection buffer to an PGF2 $\alpha$  column (containing 2  $\mu$ mole of matrix-bound PGF2 $\alpha$ ). This result shows ligand-specific elution of the pool -- a classic property of affinity-selected ligands. See Schott, H., Affinity Chromatography, (Marcel Dekker, Inc., New York), 1984.

(c) The round 6 pool was additionally characterized for PGF2 $\alpha$ -binding specificity by monitoring hydroxypropionic acid (HP)-mediated elution (HP is chemically similar to PGF2 $\alpha$ ). 0.4 ml of selection buffer containing 1.0 mM HP was added to a PGF2 $\alpha$  column saturated with radiolabelled round 6 pool. The elution profile showed that less than 1% of applied radiolabeled aptamer DNA was eluted by HP. This step was followed by application of 0.4 ml of selection buffer containing 1.0 mM PGF2 $\alpha$  using the same column, and resulted in the elution of over 95% of radiolabelled aptamer DNA from the column. This result demonstrated that the round 6 pool was binding specifically to PGF2 $\alpha$  and did not bind to a chemically similar molecule such as HP.

(d) To further characterize the round 6 pool, the pool was incubated with 5  $\mu$ mole of PGF2 $\alpha$  in selection buffer for 30 minutes at room temperature and then added to a PGF2 $\alpha$  column as described above. Less than 2% of the total cpm associated with the pool bound to the column. A PGF2 $\alpha$  column loaded with the round 6 pool in selection buffer adsorbs 75% of the input oligonucleotides (here 75% of the counts bound to the column because only 0.05 nmole of aptamer was added to the column).

(e) Analysis of the selection and elution buffers was carried out by incubating the round 6 pool

with a PGF2 $\alpha$  column by suspending the pool in selection buffer containing 20 mM EDTA to remove Mg<sup>++</sup> ions by chelation. Less than 2% of the total cpm associated with the pool bound to the column, while a control column loaded with the round 6 pool in selection buffer bound as described above (75% of the counts bound to the column because only 0.05 nmole of oligonucleotides was added to the column, resulting in a 10-fold increase in the PGF2 $\alpha$ :oligonucleotide molar ratio compared to the binding ratio used to generate the PGF2 $\alpha$  aptamer pool). This indicated that specific binding of oligonucleotides involves structural features that required the presence of Mg<sup>++</sup> ion. The use of EDTA in the elution buffer efficiently removes Mg<sup>++</sup> ion from solution and thus prohibits specific binding of oligonucleotides to the PGF2 $\alpha$  matrix.

(f) The following additional characterization method is proposed:

The round 6 pool is characterized by determining the elution profile obtained after washing a PGF2 $\alpha$  column (200  $\mu$ l support volume) saturated with the round 6 pool. The washes are carried out using 0.4 ml of selection buffer containing 1.0 mM solutions of a series of compounds that resemble PGF2 $\alpha$  more closely than HP does. In each case, the elution with a molecule similar to PGF2 $\alpha$  will be followed by elution with 0.4 ml of selection buffer containing 1.0 mM PGF2 $\alpha$  to determine the efficiency of PGF2 $\alpha$  elution. The compounds that are tested include hydroxydecanoic acid, arachidonic acid, prostaglandin A, prostaglandin B, and other eicosanoids.

Washes using chemically similar molecules are utilized for isolation of aptamers that bind to specific compounds. Elution of a PGF2 $\alpha$  column saturated with the round 6 pool using 1.0 mM 8-iso-PGF2 $\alpha$  (Cayman Chemical Company, catalog No. 16350, an isomer of PGF2 $\alpha$ ), followed

by elution with selection buffer containing 1.0 mM PGF2 $\alpha$  results in isolation of aptamers that preferentially bind to either PGF2 $\alpha$  or the 8-iso-PGF2 $\alpha$  isomer. Alternatively, columns made using equimolar amounts of PGF2 $\alpha$  and 8-iso-PGF2 $\alpha$  are used to generate a pool of aptamers containing species that bind to one or the other isomer or both. Some of these aptamers presumably bind to regions of the PGF2 $\alpha$  structure that are unaffected by the isomerization. Chemically modified eicosanoids are used in a similar manner.

### Example 3

#### Selection of Aptamers From Non-Predetermined Pools

##### 15 A. Preparation of PGF2 $\alpha$ Linked to a Solid Support

PGF2 $\alpha$  derivatized Toyopearl<sup>TM</sup> (Toyo Haas, Inc., Woburn, MA) support (charged with 10  $\mu$ moles PGF2 $\alpha$ /mL matrix) was used for all selections described. Selections were carried out according to the manufacturer's instructions. PGF2 $\alpha$  was purchased from Sigma Chemical Co. (Cat. No. P 3023) and <sup>3</sup>H-PGF2 $\alpha$  was purchased from New England Nuclear.

PGF2 $\alpha$  (salt) (10 mg) is dissolved in H<sub>2</sub>O/methanol (1 ml) and converted to the sodium salt by passage over an ion exchange column. The eluent is evaporated, dissolved in dioxane and converted to the NHS-ester by treatment with N-hydroxy-succinimide (NHS) and diisopropyl carbodiimide for 24 hrs. This mixture is then added to a toyopearl AF-amino 650M (Toyo Haas, Inc.) support (1 ml of settled support) which has been washed previously with 200 mM NaHCO<sub>3</sub>. The mixture is shaken for 24 hours and the support is washed with 200 mM NaHCO<sub>3</sub> solution. To determine the amount of loading the above-described coupling procedure is repeated except that a small amount of tritiated PGF2 $\alpha$  is added and the coupling

yield is determined from the amount of  $^3\text{H}$ -label associated with the support.

After completed PGF2 $\alpha$  coupling, the support is capped by treatment with acetic acid NHS-ester in dioxane/buffer 1:1. (The buffer is 200 mM NaHCO<sub>3</sub>). The all-capped support is made by treatment of toyopearl AF-amino 650M with acetic acid NHS-ester in the same manner as described above.

10 B. Selection of Aptamers of Substantially Non-Predetermined Sequence That Bind to PGF2 $\alpha$  Linked to Solid Support

A pool of aptamers consisting of 60 bases of completely random sequence is synthesized by standard solid phase techniques using phosphoramidite chemistry (Gait M.J., Oligonucleotide Synthesis, IRL Press, 1984; Cocuzza, A., Tetrahedron Lett. (1989) 30:6287-6291).  $1.3 \times 10^{36}$  different aptamer sequences are possible in a random 60-mer pool. A standard 1  $\mu\text{M}$  scale synthesis followed by HPLC purification yields 60 nmoles of single stranded DNA. Assuming that each base residue has an average molecular weight of 350, the synthesis yields 1.26 mg of purified DNA. The aptamers are synthesized with a phosphate group at the 5' end. The biotin residue is linked to primer using a commercially available biotin phosphoramidite conjugate (New England Nuclear, Catalog No. NEF-707) that is incorporated into the strand after solid phase DNA synthesis using standard synthesis conditions. The biotin label is incorporated into DNA according to manufacturer's recommendations.

PGF2 $\alpha$  derivatized support (charged with 10  $\mu\text{moles}$  PGF2 $\alpha$ /mL resin) is used for all selections described. 200  $\mu\text{l}$  (2  $\mu\text{mole}$  of PGF2 $\alpha$  ligand) support is poured into a 1.5 mL column housing. The support is washed with 3 mL of 20 mM Tris-Ac pH 7.4 containing 1 mM

MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 5 mM KCl and 140 mM NaCl (the "selection buffer"). Selection buffer mimics the ion and pH conditions found in the human circulatory system. A control column containing identical support is prepared in the same manner. This support is the parent matrix for attachment of selection ligand but has been capped as the acetamide to mimic the linkage used for attachment to PGF2a.

1 nmole of aptamer (doped with tracer amounts of <sup>32</sup>P-labeled species) is resuspended in 400 µl of selection buffer and heat denatured for 2 minutes at 95°C. The denatured DNA is immediately transferred to wet ice for 10 minutes. This material is applied to the control support. Flow is initiated and eluent collected. Flow-through is reapplied up to three times. At the end of the third application the column is rinsed with selection buffer. A column profile is established using <sup>32</sup>P quantitation via Cerenkov counting. Flow through material (2 to 4 column volumes) is pooled for application to the PGF2α support.

Application to PGF2α matrix is identical to that described above. After application to the column, the matrix is washed with 200 µL of selection buffer and the material reapplied to establish a column profile. The support is washed with additional selection buffer until the eluting <sup>32</sup>P material reaches a constant low level (less than about 0.2% of input DNA per 200 µL of flow through). The support then is washed with 1 mL of selection buffer containing increased NaCl (1M) until counts per 200 µL of wash are less than about 0.2% of input totals. Desired aptamer is eluted with a solution of 20 mM EDTA/60% acetonitrile (elution buffer). Specifically bound aptamers are recovered from the first 2 to 4 column volumes that are obtained after adding elution buffer. The solvent is removed in vacuo and the

material is chromatographed on a G50 Sephadex Nick column (Pharmacia, catalog no. 17-0855-02) as per the manufacturer's instructions using 10 mM Tris pH 7.5/0.1 mM EDTA/250 mM NaCl. The  $^{32}\text{P}$  fraction is then precipitated with 20  $\mu\text{g}$  of carrier glycogen (Boehringer Mannheim) and 2.5 volume absolute ethanol (dry ice 15 minutes). The DNA is pelleted at 14K, 15 minutes @ 4°C, washed with 70% ethanol and dried in vacuo.

10 C. Covalent linkage of linkers to aptamers with completely random sequences

Linkers of known sequence that serve as primers for amplification of the aptamer by PCR or other methods are covalently attached to the DNA in the aptamer pool as follows. 1.0 pmole of aptamer obtained as described in Section 1 (about 21 ng of which corresponds to about  $6.0 \times 10^{14}$  molecules) is added to a solution containing 1 nmole of linker 1 which contains 40 nucleotide residues (about 14  $\mu\text{g}$ ) and 1 nmole of linker 2 (about 14  $\mu\text{g}$ ).  
20 Linker 1, which will be ligated to the 5' end of the aptamer and consists of a pool of 256 different species, has the structure shown below. Four random sequence residues at the 5' end of strand A of linker 1 gives rise to the 256 different species. Four random sequence  
25 residues at the 3' end of strand C of linker 2 result in a pool of 256 linker 2 species.

linker 1:

3' HO-ACGCCGCGGTACTTACGC-N-N-N-N-OH 5'

strand A

30 5' biotin-TGCGGCGCCATGAATGCG-OH 3'

strand B

35

-85-

Linker 2 has the following structure.

linker 2:

5' HO-AGCGGCCGCTCTTCTAGA-N-N-N-N-OH 3'

strand C

5

3' HO-TCGCCGGCGAGAAGATCT-OPO<sub>3</sub> 5'

strand D

The linker 1 sequence: 5' GAATGC

3' CTTACG

is the recognition sequence for cutting by the  
10 restriction enzyme Bsm I, which cuts as follows (x  
denotes the cut site in each strand):

5' GAATGCNx

3' CTTACxGN

Positioning of the BspMI site as shown in linker 1  
15 permits subsequent precise removal of the attached linker  
from the aptamer after amplification. The linker 2  
sequence:

5' CTCTTC 3'

3' GAGAAG 5'

20 is the recognition sequence for cutting by the  
restriction enzyme Ear I, which cuts as follows (x  
denotes the cut site in each strand):

5' CTCTTCNx

3' GAGAAGNNNNx

25 Positioning of the Ear I site as shown in linker 2  
permits subsequent precise removal of the attached linker  
from the aptamer after amplification.

Nucleotide residues labeled N are random A, T,  
G or C residues and serve to anneal with the terminal  
30 four bases at the 5' end, linker 1, and 3' end, linker 2,  
of each aptamer. Perfect matches between the random  
linker bases and the terminal four random bases of the  
aptamer permit annealing and ligation of the linkers to  
the aptamer. The ligation reaction is carried out in a  
35 300 µl volume using 1,000 units of T4 DNA ligase (New

England Biolabs, Catalog No. 202CL) in standard reaction buffer (50 mM tris-HCl, pH 7.8 AT 20°C, 10 mM magnesium chloride, 20 mM dithiothreitol, 1 mM ATP, 50 µg/ml bovine serum albumin) at 12°C for 12 to 18 hours. The molar ratios of linker to matched aptamer end is approximately 1000:1, which drives the ligation reaction toward ligation of all aptamers in the pool. This ratio arises from input aptamer, 1 pmole, that has 0.0039 pmole of any given 4 base sequence at either end. 0.0039 nmole of either linker with a given 4 base overhang is present resulting in the 1000:1 ratio. For aptamers that have an end which participates in aptamer structure and/or binding, later rounds of selection and amplification will be enriched in species that have a nonrandom sequence at one or both ends. In this case, the ratio of specifically matched linker and aptamer will decrease by as much as 100 fold or more. Subsequent rounds of selection and amplification may then be carried out using linkers that reflect predominant aptamer end sequences to restore the ratio to a value near 1:1000. The conditions described generate aptamers with linkers covalently linked to each end. The ligation reaction generates two products. The first product is linker 1 ligated to the 5' end of the aptamer with linker 2 ligated to the 3' end of the aptamer. The second product is linker 2 ligated to itself to give a dimer. The dimers are removed by adding solid agarose-avidin support (Vector Labs, Inc. Catalog No. A2010), 2 mg of avidin per ml support with avidin linked to the support to the ligation reaction. Biotin attached to linker 1 strand A binds to the avidin solid support, permitting separation of dimers from aptamers with linkers covalently attached. The support is pelleted and washed three times in buffer (10 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) to remove dimers and unligated linkers synthesized using linker strand C as a



primer. The column is heated to 95°C to melt off the aptamer complement.

The following method to separate linker dimers from the aptamer pool alternatively may be utilized. For example, *E. coli* polymerase I (New England Biolabs, Catalog Nos. 209L or 210L) is used to synthesize aptamer and strand B complement, using strand C as the primer. The resulting duplex aptamer-linker and complement is then separated from linker dimers using an avidin column by washing at room temperature. The complement containing flanking linker is eluted by heating to 94°C and washing.

Protocols for adding linkers to only one end of an aptamer and amplifying it follow. For example, in a preferred embodiment, a pool of a very long linkers (several hundred nucleotides of known double stranded sequence having one 3' overhang (4 bases long) of random sequence at the 3' end of aptamer) is generated. This pool of linkers may be used to drive 3' ligation to the aptamer pool. Chain extension from the 3' overhang generates the aptamer complement. Standard blunt end ligation may then be used to circularize the double stranded structure. The structure then is cut using a restriction site in the linker, preferably one with an 8 base recognition sequence (to reduce the percentage of aptamers cut). Aptamers are then amplified using PCR or other known methods. In this method, the primers obviously may be placed anywhere within the large linker region, permitting amplification of only desired lengths of linker. Obviously, the long linker described above could be a replicon and directly used to generate an aptamer clone bank by transforming a desired host. For related protocols see PCR Technology, Principles and Applications for DNA Amplification, Chapter 10, p. 105-111 (Henry A. Erlich, ed. 1989) Stockton Press. For

another variation which may be adapted see Eun H-M, and Yoon, J-W., Biotechniques (1989) 7:992-997. A less preferred (because yields are lower) embodiment for attaching a linker to a single stranded aptamer would be  
5 ligation of single stranded linker to single stranded aptamer.

D. PCR Amplification of Aptamers With Flanking Primer Sequences

10 The selected DNA is amplified via PCR using the following conditions: 1 nmole of each primer, 250  $\mu$ M dNTPs (containing 20  $\mu$ Ci of dCTP, dGTP and dATP-total 60  $\mu$ Ci) in 200  $\mu$ L of 10 mM Tris pH 8.3 containing 50 mM KCl and 1.5 mM  $MgCl_2$ . The reaction is sealed with mineral  
15 oil. This reaction is put through 15 cycles of amplification. One cycle of PCR amplification is carried out by bringing the temperature to 94°C for 1 minute. This time is extended to 2 minutes for the initial denaturation step. The denaturation step is 60°C for 1  
20 minute. The hybridization step is 72°C for 1 minute and then back to 94°. After 15 cycles, the temperature is left at 72°C for 2 minutes to completely fill in all primed single stranded regions. Upon completion, the mineral oil is removed by extraction with  $CHCl_3$ . The  
25 solution is then vortexed and separated via centrifugation. The aqueous layer is removed and concentrated via n-butanol extraction-final volume 100  $\mu$ L. The  $^{32}P$  labeled DNA is then passed over a Sephadex G50 Nick column (Pharmacia) equilibrated in  
30 100 mM Tris pH 7.5/100 mM NaCl to remove unincorporated primer and dNTP's. The eluent is then applied to a 400  $\mu$ L avidin-agarose matrix (two applications results in >90% retention of the input). The matrix is extensively washed to remove contaminants and the single strand  
35 aptamers are eluted with 2 600  $\mu$ L washes of 0.15N NaOH.

The aptamer solution is neutralized with acetic acid to pH 6 and concentrated via n-butanol extraction to 40% of the initial volume. The material is precipitated with 1  $\mu$ l of a 20 mg/ml glycogen solution (Boehringer Mannheim) followed by adding 3 volumes of absolute ethanol and cooling on dry ice for 15 minutes. The DNA is pelleted, washed with 70% ethanol and dried in vacuo. The material is resuspended in selection buffer as described above. The procedure is repeated with aptamer pools from subsequent rounds of selection on PGF2 $\alpha$  columns.

#### E. Removal of Primers From the Amplified Aptamer Pool

Linker 2 is removed by digestion with Ear I (New England Biolabs, Catalog No. 528L) under recommended conditions using excess enzyme to insure complete cutting by the enzyme. Following Ear I digestion, the column is heated to 95°C for 3 minutes to denature the aptamer complement-strand molecule followed by washing in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) to remove all unbound strands.

The aptamer is removed from linker 1 strand A, which is bound to the agarose-avidin support, by suspending the support in 1 ml of BsmI restriction buffer (50 mM sodium chloride, 20 mM Tris-HCl, pH 7.4 at 20°C, 10 mM magnesium chloride, 10 mM 2-mercaptoethanol, 10  $\mu$ g/ml bovine serum albumin) and then annealing linker 1 strand A, followed by digestion with 300 units of BsmI enzyme at 65°C for 1 hour. BsmI digestion releases the aptamer from linker 1 strand B which remains bound to the support by biotin-avidin binding. The resulting pool of sequences is referred to as round 1 aptamers because the pool has been selected once for aptamers that bind to the PGF2 $\alpha$  molecule. The aptamer pool is then radiolabeled by incorporation of <sup>32</sup>P as described in Example 3-B. Alternatively, aptamers are labeled using radiolabeled

nucleotide triphosphates during PCR amplification. The DNA is precipitated with ethanol as described in Example 3-D.

5 F. Chemical Linkage of Linkers to Aptamers with completely random sequences

Linkers are covalently coupled to the 5' end of aptamers obtained from column selection as described in Examples 3-A and 3-B. Aptamer DNA is synthesized with a  
10 free amine group at the 5' end. Amine phosphoramidite monomers are used to generate the 5' terminal amine-nucleoside residue (using equal amounts of A, T, G and C monomer at the final coupling step).

After selection and elution of DNA, the aptamer  
15 DNA is coupled to primer sequences as follows. Linker is coupled to the 3' end using linker 2 described in Example 3-C. Linker (carrying biotin at the 5' end) is attached to the aptamer free 5' amine group by chemical coupling between primer oligomer DNA with a free 3'  
20 phosphate group. The reaction is carried out for 4 hours at room temperature in 0.1 M methylimidazole, pH 7.0 and 0.1 M 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride. The latter reagent acts as a water soluble condensing agent. The resulting aptamer con-  
25 tains the linkage, 5' X-O-P-O<sub>2</sub>-NH-CH<sub>2</sub>-Z 3', at the aptamer-linker junction; X is the 3' terminal residue of the linker and Z is the 5' terminal aptamer residue. Once linkers are attached at both ends of the aptamer, as described in the Examples above, PCR amplification is  
30 carried out and the DNA is attached to an avidin column. Free aptamer carrying a amino group at the 5' end is obtained by (i) digestion in excess Ear I enzyme, (ii) heat denaturing at 94°C, (iii) washing the column with TE, (iv) release of free aptamer after incubation of the  
35 column in 80% acetic acid for 4 hours at room

temperature. The aptamers are then recovered by neutralizing with base and ethanol precipitation.

G. Linkers Containing a RNA Residue at the 5' Aptamer-Primer Junction

DNA oligomers containing even a single RNA nucleotide residue are sensitive to RNases such as RNase T<sub>1</sub> or U<sub>1</sub>. T<sub>1</sub> and U<sub>1</sub> enzymes cleave specifically at guanine residues to yield two oligomers. One oligomer contains the RNA residue at the 3' end the phosphate group linked at the 3' position and the other oligomer contains a hydroxyl group at the 5' end. Cleavage of such an oligomer at the RNA residue is also possible by incubation of the oligomer in 0.1 M NaOH for 30 minutes and yields essentially the same products as the T<sub>1</sub> or U<sub>1</sub> enzymes. RNase sensitivity of DNA-RNA oligomers may be applied to selection and amplification of aptamers. Incorporation of an RNA residue (G\*) at the 3' terminal position of linker 1 strand B (5' biotin-TGCGGCGCCATGAATGCG\*-OH 3') will generate aptamer with a ribo-guanosine residue at the 5' end of the aptamer (i.e., at the primer 3' to 5' aptamer junction) when this oligomer is used to prime synthesis of aptamer using the complementary strand template. DNA polymerases have the capacity to initiate DNA synthesis from a free 3' hydroxyl group on either DNA or RNA oligomers. RNA-containing oligomer is synthesized using support bound protected G\* monomer (Milligen/Bioscience, Catalog No. GEN 067570) that is used directly in a 1  $\mu$ mole scale DNA synthesis using phosphoramidite chemistry according to manufacturer's instructions.

Aptamer strands have the following structure. G\* denotes the position of the guanine RNA residue.

5' biotin-TGCGGCGCCATGAATGCG\*N<sub>60</sub>TCTAGAAGAGCGGCCGCT-OH 3'

Aptamer selection for PGF2 $\alpha$  target would be carried out as described in Examples 3-A through 3-D. DNA primers (strands B and C as described in Example 3-C) are attached to aptamer DNA eluted from the column and  
5 amplification using strand B of linker 1 with a ribosyl G residue at the 3' end is used as primer for synthesis of the aptamer strand containing an RNA residue in amplification. Removal of the linkers and recovery of aptamer would be accomplished by the following series of  
10 steps. The RNA containing strand has 5' biotin attached.

1. Ear I digestion to remove primer sequences at the 3' end of the aptamer,
2. heating to 94°C for 2-3 minutes to denature the complementary strand,
- 15 3. washing the column to remove species released in steps 1 and 2,
4. aptamer release from the avidin column by T<sub>1</sub> RNase digestion, and
- 20 5. recovery of aptamer from the column by washing and ethanol precipitation.

The aptamer thus obtained is then used in a subsequent round of selection on a PGF2 $\alpha$  column. After recovery of aptamer from the column, DNA obtained in elution buffer washes is precipitated, and resuspended in  
25 buffer for kinase reaction and then ligated to flanking primer sequences as described. The kinase reaction prior to ligation of linkers is necessary to replace the 5' terminal phosphate group that is lost from the aptamer when T<sub>1</sub> digestion (or NaOH treatment) is carried out.

30

35

H. Selection of Aptamers With a 3' End That Does Not Participate in Target Molecule Binding or in Maintaining Aptamer Structure

Aptamers are obtained as described in the Examples above, except that after two rounds of selection and amplification using aptamers without flanking primer sequences, alternate rounds of selection and amplification are carried out with linker left on the 3' end during a subsequent round of selection. The population of aptamers thus obtained bind to the PGF2 $\alpha$  target regardless of the presence of linker at the 3' end. This population is a subset of all aptamers that bind to PGF2 $\alpha$ .

I. Selection of Aptamers With Several Bases of Known Base Sequence at One or Both Ends

Aptamer DNA is synthesized as described in Example 3-B except that the 5' terminal four bases have a known sequence to generate a pool of aptamers with the following sequence 5' PO<sub>3</sub>-AATTCN<sub>55</sub> 3'. A linker similar to linker 1 with the following structure is ligated to the 5' end of the aptamer,

3' HO-X<sub>17</sub>CTTAAG-OH 5'

5' biotin-X<sub>17</sub>G-OH 3'

and linker 2 of Example 3-C is ligated to the 3' end of the aptamer pool after elution from target molecules. Ligation of this linker to the aptamer creates a EcoRI site and cutting of the aptamer with EcoRI releases aptamer without addition or deletion of any residues. The use of restriction enzymes such as EcoRI are preferred in the 5' linker because cutting occurs on short double stranded regions that carry the recognition site (such as the double stranded region that occurs when aptamer is removed from the avidin column by restriction enzyme cutting after removal of the 3' linker and

complementary strand). Other restriction sites such as that for Hind III, or Xba I which leave a four base 5' overhang may be created and used at the 5' end to leave 5 bases of known sequence. Creation of a site for enzymes that leave either 2 (Cla I) or 0 (Pvu II) base 5' overhangs, respectively, will generate aptamers with 4 or 3 bases of known sequence at the 5' end of the aptamer. Sites created and used in this manner at the 3' end require the use of enzymes that leave a 0 (Sma I), 2 (Pvu I) or 4 (Apa I) base 3' overhang after cutting to generate aptamers with 3, 4 or 5 bases respectively at the 3' end with known sequence. If both ends of the aptamers have known sequences that constitute part of a restriction enzyme site, then the sites at the ends must differ from each other so that the linkers can be removed separately after amplification.

J. Selection of Aptamers Starting From a Pool of Aptamers That Vary in Length

Eleven pools of aptamers of random sequence are synthesized which vary in length from 50 to 60 bases for each pool. Equal molar amounts of each pool is mixed and the variable length pool is then used to select for aptamers that bind to PGF2 $\alpha$  as described in Examples 3-B through 3-E or 3-I above.

Example 4

Preparation of Aptamers Specific for Cell Surface Proteins

30

A. CD4

The human lung fibroblast-like cell line, CCD-18LU (American Type Culture Collection No. CCL205), is transfected with the human CD4 gene cloned into an expression vector. Cells stably expressing human CD4



protein are obtained by standard methods for transfecting cells and obtaining clones (see Molecular Cloning: A Laboratory Manual, Cold Springs Harbor, 1989). In this case, the bacterial neomycin phosphotransferase is

5 coexpressed from the CD4 vector, permitting selection for cells carrying the vector in the antibiotic G418 (Gibco). The resulting cell line expressing CD4 is called CD4<sup>+</sup>. A pool of aptamers consisting of 60 bases of random sequences flanked by 18 base primer sequences is obtained

10 by standard solid phase synthesis techniques ("Oligonucleotide Synthesis - A Practical Approach", ed. M.J. Gait, IRL Press 1984). Next, 0.1 to 1 nmoles of aptamer are added to 6 ml of tissue culture medium [minimum Essential medium (Eagle)] without fetal bovine

15 serum. Two confluent 10 cm tissue culture plates of CCD-18LU cells are washed twice in 5 ml medium lacking serum followed by addition of 3 ml of medium containing the aptamer pool. The plates are left at 37°C for 30 minutes. Medium from the two CCD-18LU plates is then

20 recovered and pooled.

The recovered aptamer in medium is added to 2 confluent plates of CD4<sup>+</sup> cells previously washed twice in 5 ml per wash of medium lacking serum. The plates are left at 37°C for 30 min. After incubation, the plates

25 are washed two times in medium and one time in saline using 5 ml per wash. The CD4<sup>+</sup> cells are then treated with trypsin (1.5 ml trypsin 0.01% solution in 10 mM EDTA) for 30 minutes at 37°C. The medium containing cells is briefly spun to pellet out the cells. The

30 aptamers are recovered by ethanol precipitation and amplified. The procedure is repeated 3 to 6 times to enrich for aptamers that specifically bind to the CD4 cell surface protein. Binding to CD4 is monitored by measuring the amount of radiolabeled aptamer that is

35 retained after binding to CD4<sup>+</sup> cells. Radiolabeled

aptamers are obtained by a standard kinase reaction using  $\alpha$ - $^{32}\text{P}$ -ATP to label the 5' end of aptamer after amplification. Alternatively, radiolabeled nucleoside triphosphates can be obtained by using PCR amplification to label the aptamer pool. The binding assay (positive selection) uses 0.1 nmole of labeled aptamer (approximately 3.4  $\mu\text{g}$ ) binding to one confluent plate of  $\text{CD4}^+$  cells for 30 min at 37°C, followed by two washes in medium and one saline wash. The retained radioactivity is determined by scintillation counting of cells lysed in 1 ml of 1% sodium dodecyl sulfate, 10 mM Tris pH 7.2, 10 mM EDTA. 0.05 ml of lysate is counted in a scintillation counter using standard methods and reagents (Aquasolve, New England Nuclear). Selection and amplification is continued until at least three rounds have been completed. After the third round and subsequent amplification rounds, 30-50 individual aptamers from the amplified pool are cloned and sequenced using a convenient vector such as pBluescript (Promega Biotech) and double-stranded dideoxy sequencing. Alternatively, pools of 10-20 individual clone sequences may be sequenced. When DNA sequencing reveals regions of conserved sequences, individual clones are synthesized and examined for their binding characteristics. The aptamers may be tested for their capacity to block the binding of HIV to T-cell lines such as SuptI or HUT-78 (Evans, L.A., et al., J. Immunol. (1987) 138:3415-3418) that are susceptible to infection.

Individual aptamer isolates or small pools consisting of 10 to 50 individual aptamer species that reduce HIV infectivity are used to identify optimal species for blocking HIV infectivity by interfering with the binding interaction between gp120 and CD4. Disruption of this interaction has been previously shown to reduce HIV infectivity (Clapham et al., Nature (1989)

337:368-370). After identification of optimal CD4 aptamer species, further modifications such as inclusion of covalent crosslinking base analogs (such as aziridinylcytosine) or other substituents to enhance the efficacy of the aptamer are then tested in order to further improve the aptamer for therapeutic or diagnostic uses. Lead aptamer species identified on the basis of blocking HIV infectivity are also then modified by inclusion of terminal internucleotide linkage modifications that render the oligonucleotide substantially nuclease resistant. Methods to stabilize oligonucleotides are disclosed in publication number WO90/15065, incorporated herein by reference.

15 B. HER2

HeLa cells stably transfected and expressing the gene for the HER2 oncogene referred to herein as the HER2 cell line are grown to confluency and washed two times with phosphate-buffered saline. Single-stranded oligonucleotide is generated by the random incorporation of 60 nucleotides between two primer binding sites using standard solid-phase synthesis techniques essentially as described in "Oligonucleotide Synthesis--a Practical Approach" (IRL Press 1984, ed. M.J. Gait). Approximately 5 x 10<sup>6</sup> to 1 x 10<sup>7</sup> cells are then incubated with 2 to 5 ml of tissue culture medium containing 0.1 to 1 nmoles of oligonucleotide at 37°C at a pH in the range of 7.0 to 7.4 containing between 1-5mM of divalent cations, such as magnesium or calcium. After 1-2 hours of incubation, oligonucleotides which have binding specificity for any cell surface proteins, and structure including the target HER2 glycoproteins, are then released from the cell by cleavage with trypsin (or other protease which is capable of cleaving, and thereby dissociating from cells, the protein target of interest) in buffered saline. (Evans

et al., J. Immunol. (1987) 138:3415-3418; Hoxie et al.,  
Science (1986) 234:1123-1127).

Aptamers and cell proteins released by protease  
cleavage are then digested for an additional 30 minutes  
5 at 37°C with protease to extensively degrade all cellular  
proteins. This process may be aided by a brief heat step  
(80°C for 3 minutes) followed by readdition of fresh  
protease such as pronase (Sigma Chemical Company, catalog  
no. P4914). Alternatively, a protease from the thermo-  
10 philic bacterium (Sigma Chemical Company, catalog no.  
P1512) may be used to aid recovery of aptamers from cell  
proteins. After digesting with enzymes, the aptamers  
recovered from binding to HER2 cells are recovered by  
precipitation with ethanol using glycogen as a carrier.  
15 The aptamers are then resuspended in medium (3 ml) and  
incubated with  $5 \times 10^6$  HeLa cells for about 60 minutes.  
Cellular supernatants are recovered, and the oligonu-  
cleotides precipitated from the serum-free culture medium  
after adding 200 to 800 µg glycogen (Boehringer Mannheim)  
20 followed by the addition of two volumes of ethanol.

The thus-recovered oligonucleotides, which form  
a reduced pool with cell surface protein binding  
specificity, are amplified using PCR techniques. The  
cycle is repeated 4-7 times followed by cloning of  
25 individual aptamer species. The sequences of individual  
clones are determined by standard methods. Individual  
aptamers are then synthesized and tested for binding by  
the method described in Example 4-C.

### 30 C. IL-1

The human HeLa cell line is transfected with  
two different genes to generate two lines that express  
the inserted gene. The first gene is the human IL-1  
receptor (Sims et al., Proc. Natl. Acad. Sci. (1989)  
35 86:8946-8950), giving rise to the IL-1R cell line and the

second gene is the IL-1 receptor that has been genetically engineered by standard techniques to express IL-1R that has been mutated in the extracellular domain, giving rise to the IL-1Rm cell line. Transfected clones  
5 expressing each receptor are identified by immunoprecipitation using polyclonal antibodies against the IL-1R protein.

Aptamers that specifically bind to the IL-1Rm molecule at the cell surface are obtained by selection  
10 using the IL-1Rm cell line. The procedure starts with a pool of aptamers containing 60 random bases flanked by 18 base primer sequences as described above. Two confluent plates containing about 8,000,000 IL-1R cells are incubated with a total of 0.1 nmole of aptamer in a total  
15 of 4 ml of tissue culture medium lacking serum. 0.1 nmole of aptamer pool is estimated to contain approximately  $6 \times 10^{13}$  different aptamer species, having a mass of approximately 3.4  $\mu$ g. The estimates of molecule numbers is based on the estimated molecular  
20 weight of 33,600 for a 96-mer. Each base residue in the aptamer has an average molecular weight near 350 da. The aptamer pool size may be reduced by as much as 10-fold if the initial DNA synthesis does not provide fully random sequences due to uncharacterized biases in the  
25 synthesis and purification steps.

The cells are washed three times in medium lacking serum prior to adding the aptamer pool. The IL-1R cells are incubated for 30 minutes at 37°C followed by recovery of the medium containing aptamers from the  
30 cells. The aptamers in solution are then added to washed IL-1Rm cells and incubated for 30 minutes at 37°C, followed by three washes in medium lacking serum followed by three washes in buffered saline. The cells are then trypsinized for 30 minutes at 37°C and intact cells are  
35 pelleted by a brief spin. Aptamers are recovered from

the supernatant after enzyme digestion or heating by precipitation and amplified by standard PCR methods. The process is repeated using 0.1 nmole of amplified aptamer pools at the start of each round of selection.

5           Enrichment for aptamers that specifically bind to the IL-1Rm protein is monitored by measuring the binding of selected aptamer pools to IL-1Rm cells by the following method. Aptamers obtained after 6 rounds of selection and amplification are modified according to  
10 methods disclosed herein. Biotin is covalently attached at the 5' end via linkage to N-ethyl-diethanolamine linked to the 5' nucleotide of each aptamer in the amplified pool. Alternatively, aptamers labeled for chemiluminescent detection may be synthesized and used  
15 for in situ detection of bound aptamers (Bronstein et al., Clin. Chem. (1989) 35:1856; Bronstein et al., Anal. Biochem. (1989) 180:95). Aptamers attached to target IL-1Rm molecules on IL-1Rm cells are then assayed by standard protocols using avidin and biotinylated enzymes  
20 such as alkaline or acid phosphatases. Methods for detection of nucleic acids by enzymatic methods are generally described in numerous publications (Urdea et al., Nucleic Acids Res. (1988) 16:4937-4956; Gillam, Tibtech (1987) 5:332-334). Pools containing a  
25 significant proportion of aptamers that specifically bind to the IL-1Rm target are detected by incubating a washed, confluent IL-1Rm tissue culture plate containing about  $5 \times 10^{-6}$  cells with 0.01 nmole of labeled aptamer from the selected pool mixed with 0.1 nmole of unlabeled aptamer  
30 from the initial random pool for 30 minutes at 23°C. After incubation, the plate is washed three times in buffered saline and bound labeled IL-1Rm aptamer is detected enzymatically. The presence of nitroblue tetrazolium dye (Gillam, Tibtech (1987) 5:332-334)  
35 indicates the presence of bound aptamer. Specific

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binding of selected aptamer is verified by coincubation of 0.01 nmole of labeled selected aptamer with 0.1 nmole of unlabeled selected aptamer for 30 minutes at 37°C. The unlabeled aptamer will compete with labeled aptamer and will reduce the enzyme generated dye production by about 70 to 95%. A control plate of IL-1R cells incubated with labeled selected aptamer alone and in mixture with the initial pool of unselected aptamer is included to demonstrate that binding is specific for the IL-1Rm molecule. Little or no binding of selected aptamer is observed on control IL-1R cells.

After a pool of aptamers that efficiently binds to the IL-1Rm molecule is identified, individual clones are obtained and sequenced by standard protocols (Chen et al., DNA (1985) 4:165-170). Individual aptamers are then synthesized and tested for their capacity to bind to the IL-1Rm molecule.

Aptamers that bind IL-1Rm efficiently but that do not bind to IL-1R are binding to structures in IL-1Rm that are present due to the mutation engineered into the parent IL-1R molecule. This type of selection procedure can be adapted to naturally occurring mutations, such as translocations that are correlated with pathological conditions. Protein structures uniquely associated with a mutation may be used to generate aptamers that specifically bind to those structures. Such aptamers would be useful for both diagnostic and therapeutic applications.

#### 30 D. Serum-Enhanced IL-1 Selection

Aptamers which bind to IL-1R can be obtained by following a protocol as described in Example 15 above except that HeLa is used as the control cell line and the target is the IL-1R molecule on the IL-1R cell line.

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Nonradioactive methods can be used to detect bound aptamers.

5 In a variation of this protocol, aptamer recovered from the HeLa control cells is incubated with IL-1R cells in serum-free medium for 15 minutes at 37°C, then prewarmed calf serum is added to give a final concentration of 10% and incubate an additional 15 minutes. The serum contains enzymes that degrade aptamers that are not tightly bound to target molecules. 10 The serum will enhance selection for aptamers that are not nuclease sensitive due to their tight association with IL-1R. After incubation, the cells are washed twice in medium without serum and once in saline, and aptamers are recovered and amplified.

15

#### Example 5

#### Selection of Aptamers that Bind to Factor X

##### A. Synthesis of Oligonucleotide Pool

20 DNA oligonucleotides containing a randomized sequence region were synthesized using standard solid phase techniques and phosphoramidite chemistry (Oligonucleotide Synthesis, Gait, M.J., ed. (IRL Press), 1984; Cocuzza, A., Tetrahedron Letters, (1989) 30:6287- 25 6291). A 1  $\mu$ M small-scale synthesis yielded 60 nmole of HPLC-purified single-stranded randomized DNA. Each strand consisted of specific 19-mer sequences at both the 5' and 3' ends of the strand and a random 30-mer sequence in the center of the oligomer to generate a pool of 30 68-mers with the following sequence (N = G, A, T or C):

5' TCTCCGGATCCAAGCTTATN<sub>30</sub>CGAATTCCTCGAGTCTAGA 3'

DNA 19-mers with the following sequences were used as 35 primers for PCR amplification of oligonucleotide



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sequences recovered from selection columns. The 5' primer sequence was 5' TCTCCGGATCCAAGCTTAT 3' and the 3' primer sequence was 5' biotin-O-TCTAGACTCGAGGAATTCG 3'. The biotin residue was linked to the 5' end of the 3' primer using commercially available biotin phosphoramidite (New England Nuclear, Cat. No. NEF-707). The biotin phosphoramidite is incorporated into the strand during solid phase DNA synthesis using standard synthesis conditions.

10

B. Isolation of Factor X Aptamers Using Factor X Immobilized on a Lectin Column

A pool of aptamer DNA 68 bases in length was synthesized as described in Example 5-A, and then PCR-amplified to construct the initial pool. An aliquot of the enzymatically-synthesized DNA was further amplified in the presence of  $\alpha$ -<sup>32</sup>P-dNTPs to generate labeled aptamer to permit quantitation from column fractions.

A Factor X column was prepared by washing 1 mL (58 nmole) agarose-bound concanavalin A ("Con-A") (Vector Laboratories, cat. no. AL-1003) with 20 mM Tris-acetate buffer (pH 7.4) containing 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 5 mM KCl and 140 mM NaCl (the "selection buffer") (4 x 10 mL). 1 mL of settled support was then incubated overnight at 4°C in 10 mL selection buffer containing 368 µg (6.24 nmole) Factor X (Haematologic Technologies Inc, Cat No. HCXA-0060). After shaking overnight to permit Factor X binding to the Con-A beads, the mixture was briefly centrifuged and the supernatant removed. The beads were resuspended in fresh selection buffer and transferred to a column which was then washed with selection buffer (5 x 1 mL). A column containing 1 mL of settled beads had a void volume of approximately 300 µL. A control Con-A column was prepared by adding 1 mL of settled support to

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a column followed by 5 washes of 1 mL of selection buffer.

5 Prior to application of the aptamer DNA pool to Con-A columns, the DNA was heated in selection buffer at 95°C for 3 minutes and then cooled to room temperature for 10 minutes. The pool, consisting of 100 pmole DNA in 0.5 mL selection buffer, was then pre-run on the control Con-A column at room temperature to remove species that bound to the control support. Three additional 0.5 mL  
10 aliquots of selection buffer were added and column fractions 2, 3 and 4 (0.5 mL each) were pooled and then reapplied to the column twice. The DNA in 1.5 mL selection buffer was then recovered. Approximately 16% of total input cpm were retained on the column.

15 The recovered DNA was then applied to a Con-A-Factor X column as a 0.5 mL aliquot followed by a 1.0 mL aliquot. Flow-through was retained and reapplied to the column twice. DNA added to the column on the final application was left on the column for 1 hour at room  
20 temperature. The column was then eluted with 0.5 mL aliquots of selection buffer. 0.5 mL fractions were collected and radioactivity was determined in each fraction. Radioactivity in eluted fractions 7 through 12 were low and relatively constant. After recovery of  
25 fraction 12, the column was washed with 0.5 mL aliquots of 0.1 M  $\alpha$ -methyl-mannoside (Sigma Cat. no. M-6882) in selection buffer to elute the bound Factor X along with Factor X-bound aptamers. Fractions 14 and 15 showed a  
30 significant peak of Factor X protein level, as determined spectrophotometrically by Bradford protein stain (Bio-Rad, Cat No. 500-0006). 0.085% of the input DNA eluted in these two fractions.

Aptamer DNA (Round 1 DNA) was recovered from the Factor X by phenol extraction (2 x 0.5 mL). The  
35 aqueous phase volume was reduced to about 250  $\mu$ L by n-

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butanol extraction. Aptamer DNA was precipitated on dry ice using 3 volumes of ethanol and 20  $\mu$ g of glycogen as a carrier. The DNA was pelleted, washed once in 70% ethanol and then dried.

5

C. Amplification of Factor X Selected Aptamers

Round 1 DNA from Example 5-B was resuspended in 100  $\mu$ L of H<sub>2</sub>O and amplified by PCR. A 200  $\mu$ L PCR reaction consisted of the following: 100  $\mu$ L template 96-mer DNA (approximately 0.01 pmoles); 20  $\mu$ L 10X buffer (100 mM Tris-Cl (pH 8.3), 500 mM KCl, 20 mM MgCl<sub>2</sub>); 32  $\mu$ L dNTP's (5 mM conc total, 1.25 mM each dATP, dCTP, dGTP, and dTTP); 20  $\mu$ L primer 1 (biotinylated 18-mer, 50  $\mu$ M); 20  $\mu$ L primer 2 (18-mer, 50  $\mu$ M); 6  $\mu$ L  $\alpha$ -<sup>32</sup>P-dNTP's (approximately 60  $\mu$ Ci); and 4  $\mu$ L Taq I Polymerase (20 units). The reaction was covered with 2 drops NUJOL mineral oil. A control reaction was also performed without template aptamer.

Initial denaturation was at 94°C for 3 minutes, but subsequent denaturation after each elongation reaction lasted 1 minute. Primer annealing occurred at 56°C for 1 minute, and elongation of primed DNA strands using the Taq polymerase ran at 72°C for 2 minutes, with 5-second extensions added at each additional cycle. The final elongation reaction to completely fill in all strands ran for 10 minutes at 72°C, and the reaction was then held at 4°C.

18 rounds of Taq polymerase elongation were carried out in order to amplify the selected aptamer DNA. After the reactions were completed, the aqueous layer was retrieved and any residual NUJOL oil was removed by n-butanol extraction, reducing the volume to 100  $\mu$ L. A sample may be removed from each of the aptamer and control reaction for quantitation and analytical PAGE. The amplified aptamer pool (100  $\mu$ L) was fractionated over

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a Nick column (G-50 Sephadex, equilibrated with 3 mL TE buffer (10 mM Tris-HCl (pH 7.6), 0.1 mM EDTA)) to remove unincorporated NTP's, primers, and salt. 400  $\mu$ L of TE buffer was then added to the column and the DNA pool was eluted from the column with an additional 400  $\mu$ L using TE buffer. (A sample may be removed from the eluent for quantitation and analytical PAGE.) The eluent (400  $\mu$ L) was loaded on an avidin agarose column (Vector Laboratories, Cat. No. A-2010) (500  $\mu$ L settled support, washed with 3 x 1 mL Tris/NaCl buffer (0.1 M Tris, 0.1 M NaCl, pH 7.5)). Approximately 90% of the loaded radioactivity remained on the column. The column was washed with Tris/NaCl buffer (4 x 400  $\mu$ L) and then the nonbiotinylated strand was eluted with 0.15 N NaOH (3 x 300  $\mu$ L fractions). More than 45% of the radioactivity on the column eluted in these three fractions. These fractions (900  $\mu$ L) were combined and neutralized with approximately 5.5  $\mu$ L of glacial acetic acid. The neutralized fractions were reduced to 250  $\mu$ L by speed vacuum or butanol extraction and the nucleic acids were precipitated with EtOH. The resultant pellet was dissolved in 102  $\mu$ L selection buffer. A 2  $\mu$ L sample was removed for quantitation and analytical PAGE. The resulting amplified Round 1 Pool was applied to a new Con-A-Factor X column as in Example 5-B to obtain Round 2 aptamers.

D. Characterization of Round 1 Through Round 11 Factor X Aptamers Obtained from Selection on Lectin Columns

Eleven rounds of Factor X aptamer selection and amplification were carried out using Con-A-Factor X columns as in Examples 5-B and 5-C. As shown in Table 3, the  $\alpha$ -methyl-mannoside eluate in fractions 14 and 15 contained a maximum of about 18% of input DNA at selection round 11 using the described conditions.

Table 3

	Round	% DNA eluted by $\alpha$ -methyl-mannoside*	% DNA bound to control support
5	1	0.085	14.0
	2	1.400	37.0
	3	14.000**	27.0
	4	1.800	21.0
10	5	1.100	18.0
	6	1.500	10.5
	7	.620	4.8
	8	1.100	10.6
	9	1.500	12.1
15	10	5.700	2.8
	11	17.800	19.0

\* 0.1 M  $\alpha$ -methyl-mannoside in selection buffer was added beginning at fraction 13 in each elution, and fractions 14 and 15 were retained and the DNA amplified. Fraction 16 was also included in rounds 7-11. Due to slow leeching of Factor X from the column, DNA bound to Factor X could also be seen in earlier fractions in rounds 10 and 11.

\*\* A high proportion of DNA was bound in round 3 due to a low input ratio of DNA to Factor X.

After amplification, approximately 5 picomoles of radiolabeled round 11 aptamer DNA was analyzed for specificity in a filter binding assay. In this assay, nitrocellulose filters (1 cm diameter) pre-soaked in selection buffer overnight at 4°C and DNA in 100  $\mu$ L of selection buffer was incubated at room temperature for 10 minutes with: (1) An unselected 68-mer oligonucleotide

DNA pool, (2) unselected DNA with Factor X ( $1 \mu\text{M}$ ), (3) Round 11 aptamer DNA and Factor X ( $1 \mu\text{M}$ ), and (4) Round 11 aptamer DNA alone. The filters were then washed 3 times with 3.0 mL of selection buffer at  $37^\circ$  and radioactivity was counted to determine the amount of DNA that was retained as a Factor X complex. The results are shown in Table 4.

Table 4

DNA	% DNA Bound to Filter
Unselected 68-mer	1.2
Unselected 68-mer + Factor X	1.3
Round 11 aptamer + Factor X	24.6
Round 11 aptamer	0.9

Unselected DNA did not show significant binding to the Factor X while selected aptamer DNA bound to Factor X. Binding results show specific Factor X binding. Based on the filter binding results in Table 10, a  $K_D$  of approximately  $2 \mu\text{M}$  can be estimated for the round 11 pool.

Example 6Selection of Aptamers that Bind to ThrombinA. Synthesis of Oligonucleotide Pool

DNA oligonucleotides containing a randomized sequence region were synthesized using standard solid phase techniques and phosphoramidite chemistry (Oligonucleotide Synthesis, Gait, M.J., ed. (IRL Press), 1984; Cocuzza, A., Tetrahedron Letters, (1989) 30:6287-6291). A  $1 \mu\text{M}$  small-scale synthesis yielded 60 nmole of

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HPLC-purified single-stranded randomized DNA. Each strand consisted of specific 18-mer sequences at both the 5' and 3' ends of the strand and a random 60-mer sequence in the center of the oligomer to generate a pool of  
5 96-mers with the following sequence (N = G, A, T or C):

5' HO-CGTACGGTCGACGCTAGCN<sub>60</sub>CACGTGGAGCTCGGATCC-OH 3'

DNA 18-mers with the following sequences were used as  
10 primers for PCR amplification of oligonucleotide sequences recovered from selection columns. The 5' primer sequence was 5' HO-CGTACGGTCGACGCTAGC-OH 3' and the 3' primer sequence was 5' biotin-O-GGATCCGAGCTCCACGTG-OH 3'. The biotin residue was linked  
15 to the 5' end of the 3' primer using commercially available biotin phosphoramidite (New England Nuclear, Cat. No. NEF-707). The biotin phosphoramidite is incorporated into the strand during solid phase DNA synthesis using standard synthesis conditions.

20 In another, similar experiment, a pool of 98-mers with the following sequence was synthesized:

5' HO-AGAATACTCAAGCTTGCCG-N<sub>60</sub>-ACCTGAATTCGCCCTATAG-OH 3'.

25 DNA 19-mers with the following sequences can also be used as primers for PCR amplification of oligonucleotides recovered from selection columns. The 3' primer sequence is

30 5' biotin-O-CTATAGGGCGAATTCAGGT-OH 3'

and the 5' primer sequence is

5' HO-AGAATACTCAAGCTTGCCG-OH 3'.

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It will be noted that in all cases, the duplex form of the primer binding sites contain restriction enzyme sites.

5. B. Isolation of Thrombin Aptamers Using Thrombin Immobilized on a Lectin Column

A pool of aptamer DNA 96 bases in length was synthesized as described in Example 6-A, and then PCR-amplified to construct the initial pool. A small amount  
10 of the enzymatically-synthesized DNA was further amplified in the presence of  $\alpha$ -<sup>32</sup>P-dNTPs to generate labeled aptamer to permit quantitation from column fractions.

A thrombin column was prepared by washing 1 ml  
15 (58 nmole) agarose-bound concanavalin A ("Con-A") (Vector Laboratories, cat. no. AL-1003) with 20 mM Tris-acetate buffer (pH 7.4) containing 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 5 mM KCl and 140 mM NaCl (the "selection buffer") (4 x 10 ml). 1 ml of settled support was then incubated overnight at  
20 4°C in 10 ml selection buffer containing 225 µg (6.25 nmole) thrombin (Sigma, Cat. no. T-6759). After shaking overnight to permit thrombin binding to the Con-A beads, the mixture was briefly centrifuged and the supernatant removed. The beads were resuspended in fresh selection  
25 buffer and transferred to a column which was then washed with selection buffer (5 x 1 ml). A column containing 1 ml of settled beads had a void volume of approximately 300 µL. A control Con-A column was prepared by adding 1 ml of settled support to a column followed by 5 washes of  
30 1 ml of selection buffer.

Prior to application of the aptamer DNA pool to Con-A columns, the DNA was heated in selection buffer at 95°C for 3 minutes and then cooled on ice for 10 minutes. The pool, consisting of 100 pmole DNA in 0.5 ml selection  
35 buffer, was then pre-run on the control Con-A column at



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room temperature to remove species that bound to the control support. Three additional 0.5 ml aliquots of selection buffer were added and column fractions 2, 3 and 4 (0.5 ml each) were pooled and then reappplied to the column twice. The DNA in 1.5 ml selection buffer was then recovered. Approximately 1% of total input cpm were retained on the column.

The recovered DNA was then applied to a Con-A-thrombin column as a 0.5 ml aliquot followed by a 1.0 ml aliquot. Flow-through was retained and reappplied to the column twice. DNA added to the column on the final application was left on the column for 1 hour at room temperature. The column was then eluted with 0.5 ml aliquots of selection buffer. 0.5 ml fractions were collected and radioactivity was determined in each fraction. Radioactivity in eluted fractions 7 through 12 were low and relatively constant. After recovery of fraction 12, the column was washed with 0.5 ml aliquots of 0.1 M  $\alpha$ -methyl-mannoside (Sigma Cat. no. M-6882) in selection buffer to elute the bound thrombin along with thrombin-bound aptamers. Fractions 14 and 15 showed a significant peak of thrombin enzyme activity, as determined spectrophotometrically by conversion of a chromogenic substrate (Kabi Diagnostica, Cat. no. S-2238). 0.01% of the input DNA eluted in these two fractions.

Aptamer DNA (Round 1 DNA) was recovered from the thrombin by phenol extraction (2 x 0.5 ml). The aqueous phase volume was reduced to about 250  $\mu$ l by n-butanol extraction. Aptamer DNA was precipitated on dry ice using 3 volumes of ethanol and 20  $\mu$ g of glycogen as a carrier. The DNA was pelleted, washed once in 70% ethanol and then dried.

C. Amplification of Selected Thrombin Aptamers

Round 1 DNA from Example 6-B was resuspended in 100  $\mu$ l of H<sub>2</sub>O and amplified by PCR. A 200  $\mu$ l PCR reaction consisted of the following: 100  $\mu$ l template 96-mer DNA (approximately 0.01 pmoles); 20  $\mu$ l 10X buffer (100 mM Tris·Cl (pH 8.3), 500 mM KCl, 20 mM MgCl<sub>2</sub>); 32  $\mu$ l dNTP's (5 mM conc total, 1.25 mM each dATP, dCTP, dGTP, and dTTP); 20  $\mu$ l primer 1 (biotinylated 18-mer, 50  $\mu$ M); 20  $\mu$ l primer 2 (18-mer, 50  $\mu$ M); 6  $\mu$ l  $\alpha$ -<sup>32</sup>P-dNTP's (approximately 60  $\mu$ Ci); and 2  $\mu$ l Taq I Polymerase (10 units). The reaction was covered with 2 drops NUJOL mineral oil. A control reaction was also performed without template aptamer.

Initial denaturation was at 94°C for 3 minutes, but subsequent denaturation after each elongation reaction lasted 1 minute. Primer annealing occurred at 60°C for 1 minute, and elongation of primed DNA strands using the Taq polymerase ran at 72°C for 2 minutes, with 5-second extensions added at each additional cycle. The final elongation reaction to completely fill in all strands ran for 10 minutes at 72°C, and the reaction was then held at 4°C.

18 rounds of Taq polymerase elongation were carried out in order to amplify the selected aptamer DNA. After the reactions were completed, the aqueous layer was retrieved and any residual NUJOL oil was removed by n-butanol extraction, reducing the volume to 100  $\mu$ L. A sample may be removed from each of the aptamer and control reaction for quantitation and analytical PAGE. The amplified aptamer pool (100  $\mu$ L) was run over a Nick column (G-50 Sephadex, washed with 3 mL TE buffer (10 mM Tris·HCl (pH 7.6), 0.1 mM EDTA)) to remove unincorporated NTP's, primers, and salt. 400  $\mu$ L of TE buffer was then added to the column and the DNA pool was eluted from the column with an additional 400  $\mu$ L using TE buffer. (A

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sample may be removed from the eluent for quantitation and analytical PAGE.) The eluent (400  $\mu$ L) was loaded on an avidin agarose column (Vector Laboratories, Cat. No. A-2010) (500  $\mu$ L settled support, washed with 3 x 1 mL Tris/NaCl buffer (0.1 M Tris, 0.1 M NaCl, pH 7.5)).

Approximately 90% of the loaded radioactivity remained on the column. The column was washed with Tris/NaCl buffer (4 x 400  $\mu$ l) and then the nonbiotinylated strand was eluted with 0.15 N NaOH (3 x 300  $\mu$ L fractions). More than 45% of the radioactivity on the column eluted in these three fractions. These fractions (900  $\mu$ l) were combined and neutralized with approximately 3.5  $\mu$ l of glacial acetic acid. The neutralized fractions were reduced to 250  $\mu$ l by speed vacuum or butanol extraction and the nucleic acids were precipitated with EtOH. The resultant pellet was dissolved in 102  $\mu$ l selection buffer. A 2  $\mu$ l sample was removed for quantitation and analytical PAGE. The resulting amplified Round 1 Pool was applied to a new Con-A-thrombin column as in Example 22 to obtain Round 2 aptamers.

D. Characterization of Round 1 Through Round 5 Thrombin Aptamers Obtained from Selection on Lectin Columns

Five rounds of thrombin aptamer selection and amplification were carried out using Con-A-thrombin columns as in Examples 6-B and 6-C. As shown in Table 5, the combined fractions 14 and 15 contained a maximum of about 10% of input DNA using the described conditions.

Table 5

Round	% DNA eluted by $\alpha$ -methyl-mannoside*	% DNA bound to control support
5		
1	0.01	0.7
2	0.055	1.9
3	5.80	2.3
4	10.25	1.1
5	9.70	1.0

10

\* 0.1 M  $\alpha$ -methyl-mannoside in selection buffer was added as fraction 13 in each elution, and fractions 14 and 15 were retained and the DNA amplified. Due to slow leeching of thrombin from the column, DNA bound to thrombin could also be seen in earlier fractions in rounds 3-5.

15

After amplification, round 5 aptamer DNA was analyzed for specificity in a filter binding assay. In this assay, nitrocellulose filters (1 cm diameter) prebound with salmon sperm DNA were used to bind either: (1) An unselected 96-mer oligonucleotide DNA pool, (2) unselected DNA with thrombin (60 pmole), (3) Round 5 aptamer DNA and thrombin (60 pmole), (4) Round 5 aptamer DNA alone, or (5) Round 5 aptamer DNA and ovalbumin (60 pmole). In each case 3.5 pmole of DNA was used and the incubation was in 200  $\mu$ L selection buffer at room temperature for 1 hour. The filters were then washed 3 times with 3.0 ml of selection buffer and radioactivity was counted to determine the amount of DNA that was retained as a thrombin complex. The results are shown in Table 6.

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Table 6

	DNA	% DNA Bound to Filter
5	Unselected 96-mer	0.08
	Unselected 96-mer + thrombin	0.06
	Round 5 aptamer + thrombin	20.42
	Round 5 aptamer	0.07
	Round 5 aptamer + ovalbumin	0.05
10		

Unselected DNA did not show significant binding to the thrombin while selected aptamer DNA bound to thrombin. Binding results show specific thrombin binding with no detectable ovalbumin binding.

Round 5 aptamer DNA was then amplified using the following 3' primer sequence:

5' HO-TAATACGACTCACTATAGGGATCCGAGCTCCACGTG-OH 3'

and the 5' 18-mer primer sequence shown in Example 21. The 36-mer primer was used to generate internal BamHI restriction sites to aid in cloning. The amplified Round 5 aptamer DNA was then cloned into pGEM 3Z (Promega). 32 of the resulting clones were then amplified directly using the following 5' primer sequence:

5' HO-CTGCAGGTCGACGCTAGC-OH 3'

and the 3' biotinylated 18-mer primer sequence shown in Example 21, and then sequenced.

Filter binding assays using aptamer DNA from 14 of the clones were used to determine the dissociation constants ( $K_D$ ) for thrombin as follows: Thrombin concentrations between 10  $\mu$ M and 1 nM were incubated at

room temperature in selection buffer for 5 minutes in the presence of 0.08 pmole of radiolabeled 96-mer derived from cloned Round 5 aptamer DNA. After incubation, the thrombin and aptamer mixture was applied to

5. nitrocellulose filters (0.2 micron, 2.4 cm diameter) that were pretreated with salmon sperm DNA (1 mg/ml DNA in selection buffer) and washed twice with 1 ml selection buffer. After application of thrombin mixture, the filters were washed three times with 1 ml selection

10. buffer. The radioactivity retained on the filters was then determined.  $K_D$  values for the individual clones ranged from 50 to >2000 nM.

The DNA sequence of the 60-nucleotide randomly-generated region from 32 clones was determined in order

15. to examine both the heterogeneity of the selected population and to identify homologous sequences. Sequence analysis showed each of the 32 clones to be distinct. However, striking sequence conservation was found. The hexamer 5' GGTGG 3' was found at a variable

20. location within the random sequence in 31 of 32 clones, and five of the six nucleotides are strictly conserved in all 32. Additionally, in 28 of the 32 clones a second hexamer 5' GGNTGG 3', where N is usually T and never C, is observed within 2-5 nucleotides from the first

25. hexamer. Thus, 28 clones contain the consensus sequence 5' GGNTGG(N)<sub>z</sub>GGNTGG 3' where z is an integer from 2 to 5. The remaining 4 clones contain a "close variant sequence" (a sequence differing by only a single base). A

30. compilation of the homologous sequences are shown in Figure 1. It should be noted that DNA sequencing of several clones from the unselected DNA population or from a population of aptamers selected for binding to a different target revealed no homology to the thrombin-selected aptamers. From these data we conclude that this

35. consensus sequence contains a sequence which is

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responsible either wholly or in part, for conferring thrombin affinity to the aptamers.

Clotting time for the thrombin-catalyzed conversion of fibrinogen (2.0 mg/mL in selection buffer) to fibrin at 37°C was measured using a precision coagulation timer apparatus (Becton-Dickinson, Cat. nos. 64015, 64019, 64020). Thrombin (10 nM) incubated with fibrinogen alone clotted in 40 sec, thrombin incubated with fibrinogen and P1 nuclease (Boehringer-Mannheim, Indianapolis, IN) clotted in 39 sec, thrombin incubated with fibrinogen and aptamer clone #5 (200 nM) clotted in 115 sec, and thrombin incubated with fibrinogen, clone #5 (200 nM) and P1 nuclease clotted in 40 sec. All incubations were carried out at 37°C using reagents prewarmed to 37°C. Aptamer DNA or, when present, P1 nuclease, was added to the fibrinogen solution prior to addition of thrombin. These results demonstrated that (i) thrombin activity was inhibited specifically by intact aptamer DNA and (ii) that inhibitory activity by aptamer did not require a period of prebinding with thrombin prior to mixing with the fibrinogen substrate.

Inhibition of thrombin activity was studied using a consensus-related sequence 7-mer, 5' GGTGGG 3', or a control 7-mer with the same base composition but different sequence (5' GGGGGT 3'). Clotting times were measured using the timer apparatus as above. The thrombin clotting time in this experiment was 24 sec using thrombin alone (10 nM), 26 sec with thrombin and the control sequence at 20  $\mu$ M and 38 sec with thrombin plus the consensus sequence at 20  $\mu$ M, indicating specificity for thrombin inhibition at the level of the 7-mer.

The inhibitory aptamers were active at physiological temperature under physiologic ion conditions and were able to bind to thrombin in the

presence of the fibrinogen substrate, a key requirement for therapeutic efficacy.

#### Example 7

##### Modified Thrombin Aptamers

5 Modified forms of the single-stranded, thrombin consensus sequence-containing deoxynucleotide 15-mer described in Example 7, 5' GGTGGTGTGGTTGG 3', and a  
10 closely related 17-mer, were synthesized using conventional techniques. These aptamers for the most part contain the identical nucleotide sequences, bases, sugars and phosphodiester linkages as conventional nucleic acids, but substitute one or more modified  
15 linking groups (thioate or MEA), or modified bases (uracil or 5-(1-pentynyl)-2'-deoxy)uracil). The aptamers containing 5-(1-pentynyl)-2'-deoxyuridine were generated by replacing thymidine in the parent aptamers. Thrombin aptamers containing 5-(1-pentynyl)-2'-deoxyuridine were also obtained by selection as described in Examples 13  
20 and 14 below.

Independent verification of the  $K_1$  for the nonmodified 15-mer was made by determining the extent of thrombin inhibition with varying DNA concentration. The data revealed 50% inhibition of thrombin activity at  
25 approximately the same concentration as the derived  $K_1$ , strongly suggesting that each bound thrombin was largely, if not completely, inhibited, and that binding occurred with a 1:1 stoichiometry.

30

35



Table 7

	Compound	K <sub>1</sub> (nM)
	GGTTGGTGTGGTTGG	20
5	GGTTGGTGTGGTTGG <sup>#</sup> G <sup>*</sup> T	35
	GGTTGGTGTGGTT <sup>*</sup> G <sup>*</sup> G	40
	G <sup>*</sup> G <sup>*</sup> T <sup>*</sup> T <sup>*</sup> G <sup>*</sup> G <sup>*</sup> T <sup>*</sup> G <sup>*</sup> T <sup>*</sup> G <sup>*</sup> T <sup>*</sup> G <sup>*</sup> G	280
	GGTTGG (dU) G (dU) GGTTGG	15
	GG (dU) TGGTGTGG (dU) TGG	80
10	GGTTGGTGTGGTU'GG	20
	* indicates a thioate (i.e., P(O)S) linkage	
	# indicates a MEA linkage	
	U' indicates 5-(1-pentynyl)uracil	
15		

Example 8

Incorporation of 5-(1-pentynyl)-2'-deoxyuridine  
Into Aptamer Candidate DNA

5-(1-pentynyl)-2'-deoxyuridine was synthesized and converted to the triphosphate as described in Otvos, L., et al., Nucleic Acids Res (1987) 1763-1777. The pentynyl compound was obtained by reacting 5-iodo-2'-deoxyuridine with 1-pentyne in the presence of palladium catalyst. 5-(1-pentynyl)-2'-deoxyuridine triphosphate was then used as a replacement for thymidine triphosphate in the standard PCR reaction.

A pool of 96-mer single-stranded DNA was synthesized, each strand consisting of specific 18-mer PCR primer sequences at both the 5' and 3' ends and a random 60-mer sequence in the center of the oligomer. Details of synthesis of the pool of single-stranded DNA is disclosed in Examples 1-6 above. PCR conditions were the same as those described above, with the following changes. dATP, dGTP and dCTP were all used at a

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concentration of 200  $\mu$ M. The optimal concentration for synthesis of full-length 96-mer DNA via PCR using 5-(1-pentynyl)-2'-deoxyuridine was 800  $\mu$ M. Generation of PCR-amplified fragments demonstrated that the Taq polymerase both read and incorporated the base as a thymidine analog. Thus, the analog acted as both substrate and template for the polymerase. Amplification was detected by the presence of a 96-mer band on an EtBr-stained polyacrylamide gel.

10

#### Example 9

##### Incorporation of Other Base Analogs Into Candidate Aptamer DNA

Ethyl, propyl and butyl derivatives at the 5-position of uridine, deoxyuridine, and at the N<sup>4</sup>-position of cytidine and deoxycytidine are synthesized using methods described above. Each compound is converted to the triphosphate form and tested in the PCR assay described in Example 1 using an appropriate mixture of three normal deoxytriphosphates or ribotriphosphates along with a single modified base analog.

This procedure may also be performed with N<sup>6</sup>-position alkylated analogs of adenine and deoxyadenine, and the 7-position alkylated analogs of deazaguanine, deazadeoxyguanine, deazaadenine and deazadeoxyadenine, synthesized using methods described in the specification.

#### Example 10

##### Thrombin Aptamer Containing Substitute Internucleotide Linkages

Modified forms of the 15-mer thrombin aptamer, 5' GGTGGTGTGGTTGG 3' containing one or two formacetal internucleotide linkages (O-CH<sub>2</sub>-O) in place of the phosphodiester linkage (O-PO(O<sup>-</sup>)-O) were synthesized and

35

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assayed for thrombin inhibition as described above. The H-phosphonate dimer synthon was synthesized as described in Matteucci, M.D., Tet. Lett. (1990) 31:2385-2387. The formacetal dimer, 5' T-O-CH<sub>2</sub>-O-T 3', was then used in solid phase synthesis of aptamer DNA. Control unmodified aptamer DNA was used as a positive control. The results that were obtained are shown in Table 8.

Table 8

Compound	clot time (sec)		
	100 nM	20 nM	0 nM
GGT•TGGTGTGGTTGG	105	51	--
GGTTGGTGTGGT•TGG	117	48	--
GGT•TGGTGTGGT•TGG	84	60	--
GGTTGGTGTGGTTGG	125	49	--
NO DNA CONTROL	--	--	25

• indicates a formacetal linkage

Example 11

Thrombin Aptamer Containing Abasic  
Nucleotide Residues

Modified forms of the 15-mer thrombin aptamer, 5' GGTTGGTGTGGTTGG 3' containing one abasic residue at each position in the aptamer were synthesized and assayed for thrombin inhibition as described above. The abasic residue, 1,4-anhydro-2-deoxy-D-ribitol was prepared as described in Eritja, R., et al, Nucleosides and Nucleotides (1987) 6:803-814. The N,N-diisopropylamino cyanoethylphosphoramidite synthon was prepared by standard methods as described in Caruthers, M.H. Accounts Chem. Res. (1991) 24:278-284, and the derivatized CGP support was prepared by the procedures described in Dahma, M.J., et al, Nucleic Acids Res. (1990) 18:3813.

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The abasic residue was singly substituted into each of the 15 positions of the 15-mer. Control unmodified aptamer DNA was used as a positive control. The results that were obtained are shown in Table 9.

5

Table 9

Compound	clot time (sec)	
	100 nM	0 nM
10 GGTGGGTGTGGTTGX	27	-
GGTGGGTGTGGTTXG	27	-
GGTGGGTGTGGTXGG	27	-
GGTGGGTGTGGXTGG	56	-
GGTGGGTGTGXTTGG	27	-
15 GGTGGGTGTGXGTTGG	29	-
GGTGGGTGXGGTTGG	43	-
GGTGGTGTGTTGG	51	-
GGTGGXGTGGTTGG	161	-
GGTGTGTGGTTGG	27	-
20 GGTGXGTGTGGTTGG	27	-
GGTXGGTGTGGTTGG	27	-
GGXTGGTGTGGTTGG	62	-
GXTTGGTGTGGTTGG	27	-
XGTTGGTGTGGTTGG	28	-
25 GGTGGTGTGGTTGG	136	-
NO DNA CONTROL	-	26

X - indicates an abasic residue

30

Example 12Thrombin Aptamers Containing 5-(1-Propynyl)-2'-deoxyuridine Nucleotide Residues

Modification of the 15-mer thrombin aptamer, 5'

GGTGGTGTGGTTGG 3' to contain 5-(1-propynyl)-2'-

35 deoxyuridine nucleotide analogs at the indicated

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positions in the aptamer was effected by the synthesis of these aptamers. They were assayed for thrombin inhibition as described above. The aptamer and the H-phosphonate were prepared as described in DeClercq, E., et al, J. Med. Chem. (1983) 26:661-666; Froehler, B.C., et al, Nucleosides and Nucleotides (1987) 6:287-291; and Froehler, B.C., et al, Tet. Lett. (1986) 27:469. This analog residue was substituted at the indicated positions and the aptamer assayed for inhibition of thrombin. The results that were obtained are shown in Table 10.

Table 10

Compound	clot time (sec)	
	100 nM	0 nM
GGTTGGTGTGGTZGG	147	-
GGTTGGTGTGGZTGG	129	-
GGTTGGTGZGGTTGG	120	-
GGTTGGZGTGGTTGG	118	-
GGTZGGTGTGGTTGG	187	-
GGZTGGTGTGGTTGG	138	-
GGTTGGTGTGGTTGG	125	-
NO DNA CONTROL	-	23

Z - indicates a 5-propynyl-2'-deoxyuridine residue

Example 13Incorporation of 5-(1-pentynyl)-2'-deoxyuridineInto Aptamer Candidate DNA

5-(1-pentynyl)-2'-deoxyuridine was synthesized and converted to the triphosphate as described in Otvos, L., et al., Nucleic Acids Res (1987) 1763-1777. The pentynyl compound was obtained by reacting 5-iodo-2'-deoxyuridine with 1-pentyne in the presence of a palladium catalyst. 5-(1-pentynyl)-2'-deoxyuridine

triphosphate was then used as a replacement for thymidine triphosphate in the standard PCR reaction.

5 A pool of 60-mer single-stranded DNA was synthesized, each strand consisting of specific 18-mer PCR primer sequences at both the 5' and 3' ends and a random 20-mer sequence in the center of the oligomer. Details of synthesis of the pool of single-stranded DNA is disclosed in Example 1.

10 Because of the poor substrate activity of pentynyl dUTP when used with TAQ polymerase, VENT<sup>™</sup> thermostable polymerase, (New England Biolabs, Cat. No. 254) was employed. Amplification was performed as per the manufacturers instructions. Pentynyl dUTP was included in the reaction as a substitute for dTTP. The  
15 single-stranded 60-mer was isolated by a modification of standard procedures. The 200  $\mu$ L PCR amplification reaction was divided into two samples which were applied to two NICK<sup>™</sup> columns equilibrated (5 mL) as described. The eluent was collected, pooled and applied to avidin-  
20 agarose as described. This column was washed with buffer followed by elution of single-stranded 60-mer DNA with 0.15 N NaOH, pooled and neutralized with glacial acetic acid. Single-stranded 60-mer DNA was desalted on a NAP5  
25 column equilibrated in 20 mM Tris OAc (pH 7.4). 10X selection buffer salts were added to the sample, heated to 95°C for 3 minutes, and transferred to wet ice for 10 minutes.

#### Example 14

#### 30 Isolation of Thrombin Aptamers Using DNA Containing 5-(1-Pentynyl)-2'-deoxyuridine

The pool of aptamer DNA 60 bases in length was used essentially as described in Example 13. The aptamer pool sequence was

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5' TAGAATACTCAAGCTTCGACG-N<sub>20</sub>-AGTTTGGATCCCCGGGTAC 3',

while the 5' primer sequence was

5' TAGAATACTCAAGCTTCGACG 3'

and the 3' biotin-linked primer was

5 GTACCCGGGGATCCAAACT 3'.

Thrombin immobilized on a Con-A lectin column served as the target as described.

After five rounds of selection, aptamer DNA was recovered and amplified using thymidine triphosphate (dTTP) in place of 5-(1-pentynyl)-2'-deoxyuridine in order to facilitate subsequent cloning and replication of aptamer DNA in *E. coli*. At this stage, the presence of a thymidine nucleotide at a given location in an aptamer corresponded to the location of a 5-(1-pentynyl)-2'-deoxyuridine nucleotide in each original round five aptamer. Thus, dTTP served to mark the location of 5-(1-pentynyl)-2'-deoxyuridine residues in the original selected DNA pools.

The round five amplified DNA containing dTTP was digested with BamHI and HindIII and cloned into the corresponding sites of pGEM 3Z (Promega Biotech) and transformed into *E. coli*. DNA from 21 clones was analyzed by dideoxy sequencing. Three of the clones contained aptamer sequences that were identical. Only one of the 21 clones contained a sequence that closely resembled the original 5' GGTGG 3' binding motif obtained using thymine in the selection protocol.

One of these two clones (#17) and the original unselected pool was analyzed for thrombin binding by nitrocellulose filter assay described above using DNA labeled with <sup>32</sup>P to permit analysis of thrombin binding characteristics. The labeled DNA was synthesized by PCR and contained 5-(1-pentynyl)-2'-deoxyuridine in order to retain the original selected DNA structures. The DNA was incubated with thrombin at various concentrations between

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10 nM and 10  $\mu$ M to obtain the  $K_D$  values for thrombin binding. The  $K_D$  of the unselected pool was  $>10 \mu$ M while the  $K_D$  of clone 17 was 300 nM.

5 Radiolabeled clone 17 DNA was synthesized using thymidine in place of 5-(1-pentynyl)-2'-deoxyuridine and the resulting DNA had a  $K_D$  of  $>10 \mu$ M, demonstrating that the 5-(1-pentynyl)-2'-deoxyuracil heterocycle could not be replaced by thymine in the selected aptamer without loss of binding affinity.

10 Representative sequences that were obtained are as follows.

5' TAGTATGTATTATGTGTAG 3'  
5' ATAGAGTATATATGCTGTCT 3'  
5' GTATATAGTATAGTATTGGC 3'  
15 5' AGGATATATGATATGATTCGG 3'  
5' TACTATCATGTATATTACCC 3'  
5' CATTAAACGCGAGCTTTTTG 3'  
5' CTCCCATAATGCCCTAGCCG 3'  
5' GACGCACCGTACCCCGT 3'  
20 5' CACCAAACGCATTGCATTCC 3'  
5' GTACATTTCAGGCTGCCTGCC 3'  
5' TACCATCCCGTGGACGTAAC 3'  
5' GACTAAACGCATTGTGCCCC 3'  
5' AACGAAGGGCACGCCGGCTG 3'  
25 5' ACGGATGGTCTGGCTGGACA 3'

#### Example 15

#### Isolation of Thrombin Aptamers Using 30 DNA Containing 5-Methyl-2'-deoxycytidine

5-methyl-2'-deoxycytidine triphosphate was obtained commercially (Pharmacia, Cat. No. 27-4225-01) and used to synthesize DNA containing random sequences 60 bases in length flanked by primers 19 bases in length.  
35 The pool of aptamer DNA 98 bases in length was used



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essentially as described in Example 6. Thrombin immobilized on a Con-A lectin column served as the target as described.

Briefly, a 200  $\mu$ L PCR reaction was set up  
5 using: 10 mM Tris-HCl, pH 8.3 at 25° C, 1.5 mM MgCl<sub>2</sub>, 50 mM NaCl and 200  $\mu$ M of each of dATP, dGTP, dTTP and 5-methyl-2'-deoxycytidine triphosphate. 20  $\mu$ Ci each of  $\alpha$ -<sup>32</sup>P-dATP and dGTP were added to label the DNA. 1 nmole of 5' and 3' primer were added followed by addition of  
10 0.2 pmole of 98-mer template pool DNA. Amplification was initiated by addition of 2  $\mu$ L (10 U) of Taq polymerase followed by sealing of the reaction with a mineral oil overlay. About 16 cycles of amplification were performed followed by a 10 minute final extension to complete all  
15 duplex synthesis.

Amplified DNA was recovered (100  $\mu$ L aqueous phase), n-butanol extracted (650  $\mu$ L) and applied to a Nick column prewashed with 5 mL of buffer containing 100 mM Tris-HCl pH 7.5 and 100 mM NaCl. Eluted DNA was  
20 applied to a 0.5 mL avidin-agarose column prewashed in the same buffer and washed until DNA loss from the column was < 1000 cpm. Single stranded DNA was eluted from the avidin column by washing with 0.15 N NaCl and the eluate was neutralized to pH 7.0 using glacial acetic acid. The  
25 98-mer DNA was exchanged into selection buffer on a second Nick column and, after heat denaturation for 3 min at 95° C followed by cooling on ice for 10 min, used in aptamer selection on thrombin lectin columns. 1 mL thrombin columns were equilibrated in selection buffer  
30 prior to addition of single-stranded DNA. The single-stranded DNA was recirculated for three complete passes. Upon completion of the third pass the peak radioactive element was then applied to a 1 mL ConA/thrombin column (charged with 3 nmoles of thrombin). Radioactive single-  
35 stranded 98-mer was applied three times to this matrix.

At the third application, the column was stoppered and allowed to stand for 1 hr. The column was then washed with selection buffer and 0.5 mL aliquot fractions collected. A total wash volume of 6 mL was employed. At this time, 0.1 M  $\alpha$ -methyl-mannoside in selection buffer was then added, followed by a 4 mL total volume wash. Thrombin enzymatic activity was detected via chromogenic substrate monitored by absorbance at 405 nm. Peak thrombin fractions were pooled, extracted with phenol, and the volume reduced by nBuOH extraction. 20  $\mu$ g glycogen was added, the single-stranded 98-mer precipitated via ethanol addition and pelleted via centrifugation. The pelleted DNA was resuspended in water and used as a template for PCR amplification. This protocol was repeated to obtain a pool of DNA that resulted from 5 rounds of selection on thrombin columns.

Double-stranded DNA was digested with EcoRI and HindIII and cloned into pGEM3Z. Aptamers were then transformed into *E. coli* and analyzed by dideoxy sequencing. Round five aptamer pool DNA bound to thrombin with a  $K_D$  of approximately 300 nM.

#### Example 16

##### Demonstration of Aptamer Specificity for Binding to and Inhibition of Thrombin

The specificity of aptamer binding was demonstrated using  $^{32}$ P radiolabeled DNA and a series of proteins. To determine the binding specificity of the thrombin aptamer, 96-mer clone #29, having the partial sequence 5' CGGGGAGAGGTTGGTGTGGTTGGCAATGGCTAGAGTAGTGAC GTTTTCGCGGTGAGGTCC 3' was used. The consensus sequence is shown underlined. In addition, a 21-mer aptamer, 5' GGTGGGCTGGTTGGGTTGGG 3' was tested for inhibition of another fibrinogen-cleaving enzyme ancrod, which was obtained commercially (Sigma, Cat. No. A-5042). The

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21-mer had a  $K_I$  for thrombin of about 100 nM and its  $K_D$  was about 350 nM. Clone #29 had a  $K_D$  of about 200 nM for thrombin.

The aptamer was shown to specifically bind to thrombin by a filter binding assay. Briefly, radiolabeled aptamer DNA at about a concentration of about 1 nM was incubated with the indicated protein for several minutes at room temperature, followed by filtration of the aptamer-protein mixture through a nitrocellulose filter. The filter was washed with 3 mL of selection buffer and then radioactivity bound to the filters was determined as a % of input radioactivity. Results obtained are shown in Table 11. Binding data is shown for both unselected 96-mer DNA and for two separate experiments with clone #29 96-mer. All proteins were tested at about 1  $\mu$ M concentration except human serum albumin which was used at 100  $\mu$ M. The results that were obtained demonstrated that the 96-mer specifically bound to thrombin and had little affinity for most of the other proteins tested.

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Table 11

	<u>Protein</u>	<u>Input CPM</u>	<u>Bound CPM</u>	<u>% Bound</u>
	<u>Unselected DNA</u>			
5	Control	75573	230	0
	Thrombin	74706	6732	9.0
	Prothrombin	75366	183	<0.5
	Albumin	76560	1851	2.0
	Chymotrypsin	75566	225	<0.5
10	Trypsin	73993	306	<0.5
	Kallikrein	76066	122	<0.5
	Plasmin	74513	3994	5.0
	<u>Clone 29 DNA</u>			
15	Control	81280	126	0
	Thrombin	81753	48160	59.0
	Prothrombin	81580	8849	11.0
	Albumin	85873	1778	2.0
	Chymotrypsin	82953	207	<0.5
20	Trypsin	75673	318	<0.5
	Kallikrein	84013	143	<0.5
	Plasmin	82633	12323	15.0
	TPA	81960	192	<0.5
25	<u>Clone 29 DNA</u>			
	Control	81886	917	0
	Thrombin	82940	48796	59.0
	Prothrombin	91760	8719	9.5
	Albumin	92473	234	<0.5
30	Chymotrypsin	97060	186	<0.5
	Trypsin	97846	429	<0.5
	Kallikrein	95053	1275	<0.5
	Plasmin	66565	9704	15.0
	TPA	98166	644	<0.5
35				

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The thrombin 21-mer ancrod assay was conducted as follows. Ancrod was suspended in sterile water at a concentration of 44 U/mL. 10  $\mu$ L ancrod solution was added to 95  $\mu$ L of selection buffer prewarmed to 37°C. 5 100  $\mu$ L of this mixture was transferred to the coagulation cup of the fibrometer described above, followed by addition of 200  $\mu$ L of fibrinogen and 20  $\mu$ L of 21-mer DNA (both prewarmed to 37°C). TE buffer pH 7.0 was used as a control lacking DNA. The control clot time was 25 10 seconds while the clot time in the presence of 500 nM 21-mer was 24 seconds and was 26 seconds in the presence of 33  $\mu$ M 21-mer. This result demonstrated the specificity on inhibition of fibrinogen cleavage was limited to thrombin; ancrod was not affected.

15

#### Example 17

##### Thrombin Aptamer Pharmacokinetic Studies

A 15-mer single-stranded deoxynucleotide, 5' GGTGGTGTGGTTGG 3', identified as a consensus sequence from 30 thrombin aptamer clones as described in Example 6 20 above, was used. Young adult rats of mixed gender and strain were used. The animals were anaesthetized and a diester of the 15-mer was injected through a catheter in 200  $\mu$ l volumes (in 20 mM phosphate buffer, pH 7.4, 0.15 M 25 NaCl) at two concentrations, so that the final concentration of 15-mer in the blood was about 0.5 and 5.0  $\mu$ M respectively, although the exact concentration depends on the volume of distribution (which is unknown for this oligonucleotide). These values are 10 to 100 30 times greater than the human in vitro  $K_d$  value. No heparin was used for catheterization.

At 0, 5, 20 and 60 minutes, blood was withdrawn from the animals (approx. 500  $\mu$ l aliquots), transferred into tubes containing 0.1 volume citrate buffer, and 35 centrifuged. Rat plasma was removed and tested in a

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thrombin clotting-time assay. Six animals were used at each concentration, and three animals were injected with the control carrier solution containing no 15-mer.

A prolonged clotting time was observed at the 5 minute time point at both concentrations, with the most significant prolongation occurring at the higher dose concentration. Little or no activity was observed at 20 minutes. Thus, the 15-mer in blood withdrawn from rats 5 minutes post-injection was able to inhibit exogenously added human thrombin. A separate APTT test at the 5 minute time point showed that the 15-mer also inhibited rat blood coagulation, presumably by inhibiting rat thrombin to a significant degree. The half-life of the 15-mer in rats appears to be about 2 minutes or less.

15

#### Example 18

##### Thrombin Aptamer Primate Studies

Two thrombin aptamers were administered to adult male cynomologous monkeys. Unsubstituted 15-mer DNA with the sequence 5' GGTTGGTGTGGTTGG 3' and an analog, 5' GGTTGGTGTGGTT\*G\*G 3', containing thioate internucleotide linkages at the indicated positions (\*), were used. Aptamer was delivered as an intravenous bolus or infusion and then blood samples were withdrawn at various times after delivery of the bolus or during and after infusion. The catheter was heparinized after the 10 minute timepoint. The animals were not systematically heparinized.

Thrombin inhibition was measured by a prothrombin time test (PT) using a commercially available kit, reagents and protocol (Sigma Diagnostics, St. Louis, catalog Nos. T 0263 and 870-3). Inhibition of thrombin was indicated by an increased clot time compared to the control in the PT test. Clot times were obtained by withdrawing a sample of blood, spinning out red cells and

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using the plasma in the PT test. Control thrombin PT clot time values were obtained several minutes prior to administration of aptamer. Briefly, the PT assay was conducted using 0.1 mL of monkey plasma prewarmed to 37°  
5 C and 0.2 mL of a 1:1 mixture of thromboplastin (used according to manufacturers instructions) and  $\text{CaCl}_2$  (25 mM), also prewarmed to 37°C. Thrombin clot times were measured with a fibrometer as described above.

The animals were at least two years old and  
10 varied in weight from 4 to 6 kg. Doses of aptamer were adjusted for body weight. Aptamer DNA was dissolved in sterile 20 mM phosphate buffer (pH 7.4) at a concentration of 31.8 to 33.2 mg/mL and diluted in sterile physiological saline prior to delivery. Bolus  
15 injections were administered to give a final concentration of 22.5 mg/Kg (1 animal) of the diester aptamer or 11.25 mg/Kg (1 animal) of the diester aptamer. Infusions were administered over a 1 hour period to three groups of animals: (i) 0.5 mg/kg/min of diester 15-mer (4  
20 animals), (ii) 0.1 mg/kg/min of diester 15-mer (2 animals) and (iii) 0.5 mg/kg/min of thioate analog 15-mer (2 animals).

PT assay results from the bolus injections showed thrombin inhibition times of 7.8, 3.3 and 1.35  
25 times control at 2.5, 5.0 and 10.0 min respectively after delivery of the aptamer for the high dose animal. Inhibition times of 5.6, 2.2 and 1.2 times control were obtained from the low dose animal at the same time points.

30 Figure 2 shows a plot of the PT times from the 4 animals that received the high dose diester infusion compared to pretreatment control values. The data points show the PT clot time as an average value obtained from the 4 animals in the group. The arrows indicate time  
35 points at the beginning and end of the infusion period.

Thrombin inhibition peaked at about 10 to 20 min after the infusion was initiated and remained level until the infusion period was terminated. Inhibitory activity decreased rapidly after the infusion of aptamer terminated.

High dose diester and high dose thioate animals showed comparable inhibition of thrombin-mediated clotting, with the high dose thioate giving a sustained clot time of 2.5 to 2.7 times the control value during the course of the infusion. The low dose diester compound gave a clot time of 1.4 to 1.5 times the control value. These results demonstrated the efficacy of the native and thioate analog aptamers in primates.

Example 19  
Inhibition of Extracorporeal Blood Clotting  
By Thrombin Aptamer

Anticoagulation of a hemodialysis filter was demonstrated using the 15-mer 5' GGTGGTGTGGTTGG 3' thrombin aptamer with human blood. A bolus of 15-mer DNA was delivered to human blood at 37°C to give an aptamer concentration of 10 $\mu$ M. The blood was contained in an extracorporeal hemodialysis circuit (Travenol, Model No. CA-90). Pressure proximal to the hemodialysis filter was monitored to determine the time after administration of aptamer that coagulation occurred. Blood coagulation was marked by a pressure increase from about 50 mm Hg observed with uncoagulated blood (blood flow rate 200 mL/min) to pressure of at least 400 mm Hg.

Using citrated whole blood (recalcified at time zero), coagulation occurred at about 9 minutes after fresh blood was placed in the hemodialysis unit and circulation was begun. (In a repeat of this control experiment, coagulation occurred at 11 minutes.) A heparin control (1 U/mL) gave sustained anticoagulation



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until the experiment was terminated at 80 minutes after  
start of circulation in the unit. Blood coagulation  
occurred at 51 minutes in one trial with the 15-mer. In  
a second trial, coagulation did not occur during the 80  
5 minute course of the experiment.

Thus, methods for obtaining aptamers that  
specifically bind serum proteins such as thrombin and  
Factor X, eicosanoids, kinins such as bradykinin, and  
cell surface ligands are described, as well as the  
10 therapeutic utility of these aptamers and the use of the  
aptamers in the detection and isolation of such  
substances. Although preferred embodiments of the  
subject invention have been described in some detail, it  
is understood that obvious variations can be made without  
15 departing from the spirit and scope of the appended  
claims.

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A. Extracellular Proteins:

lipoprotein lipase  
lecithinlist-cholesterol acyl transferase  
apolipoprotein A-1  
apolipoprotein II  
apolipoprotein IV  
apolipoprotein B-48  
apolipoprotein B-100  
apolipoprotein CI  
apolipoprotein CII  
apolipoprotein CIII  
apolipoprotein D  
apolipoprotein E  
insulin  
insulin-like growth factors I and II  
angiotensin I  
angiotensin II  
renin  
angiotensin converting enzyme  
atrial natriuretic peptide  
immunoglobulin IgA constant region  
immunoglobulin IgG constant region  
immunoglobulin IgE constant region  
immunoglobulin IgM constant region  
immunoglobulin light chain kappa  
immunoglobulin light chain lambda  
immunoglobulin IgG Fc portion  
immunoglobulin IgM Fc portion  
immunoglobulin IgE Fc portion  
amyloid protein  
beta- amyloid protein  
substance P  
leu-enkephalin  
met-enkephalin  
somatostatin  
interleukin-1  
interleukin-2  
interleukin-3  
interleukin-4  
interleukin-5  
interleukin-6  
interleukin-7  
interleukin-8  
interleukin-9  
interleukin-10  
interleukin-11  
interleukin-12  
interleukin-13  
colony stimulating factor-macrophage  
colony stimulating factor-granulocyte  
colony stimulating factor-macrophage/granulocyte  
erythropoietin

myelin basic protein  
carcinoembryonic antigen  
collagen type I  
collagen type II  
collagen type III  
collagen type IV  
collagen type V  
vitronectin  
fibronectin  
fibrinogen  
albumin  
aminopeptidase  
amylase  
avidin  
B-cell growth factor  
Bence-Jones protein  
prothrombin (Factor II)  
thrombin (Factor IIa)  
tissue factor (Factor III)  
proaccelerin (Factor V)  
accelerin (Factor Va)  
proconvertin (Factor VII)  
antihemophilic factor (Factor VIII)  
Christmas factor (Factor IX)  
Stuart factor (Factor X)  
plasma thromboplastin antecedent (Factor XI)  
Hageman factor (Factor XII)  
Fibrin-stabilizing factor (Factor XIII)  
prekallikrein  
high molecular weight kininogen  
bradykinin  
kinins  
calcitonin  
carboxypeptidase A  
carboxypeptidase B  
carboxypeptidase C  
catalase  
ceruloplasmin  
cholinesterase  
chymotrypsin  
lipase  
amylase  
collagenase  
complement protein C1q  
complement protein C1r2  
complement protein C1s2  
complement protein C2  
complement protein C2a  
complement protein C2b  
complement protein C3 convertase  
complement protein C3  
complement protein C3b  
complement protein C4  
complement protein C4a

complement protein C4b  
complement protein C5a  
complement protein C5  
complement protein C5b  
complement protein C5 convertase  
complement protein C6  
complement protein C7  
complement protein C8  
complement protein C9  
complement protein lytic complex  
adrenocorticotrophic hormone  
corticotropin releasing hormone  
pepsin A  
pepsin B  
pepsin C  
trypsin  
elastase  
enterokinase  
leucine aminopeptidase  
aminotripeptidase  
dipeptidase  
prolidase  
prolinase  
alpha-glucosidase  
sucrase  
maltase  
beta-galactosidase  
oligo glucosidase  
lipase  
phospholipase A and B  
cholesterol esterase  
monoacylglycerol lipase  
carboxylic acid esterase  
alkaline phosphatase  
elastin  
myelin protein A1  
proenkephalin  
enteropeptidase  
L-asparaginase  
E-asparaginase  
epidermal growth factor  
fibrin  
fibrinopeptide A  
fibrinopeptide B  
filaggrin  
follicle-stimulating hormone  
follicle-stimulating hormone releasing hormone  
gastrin releasing peptide  
growth hormone  
glucagon  
leutinizing hormone  
leutinizing hormone releasing hormone  
human menopausal gonadotropin  
prolactin

chorionic gonadotropin  
growth hormone releasing hormone  
hemosiderin  
placental lactogen  
inhibin  
kallikrein  
acid keratin  
vimentin  
desmin  
glial fibrillary acidic protein  
leukokinin  
leupeptin  
luciferase  
melanin  
melanotropin  
melatonin  
melanotropin release inhibiting hormone  
nerve growth factor  
oxytocin  
vasopressin  
neurophysin  
neurotensin  
beta-endorphin  
adrenorphin  
dynorphin  
alpha neoendorphin  
phospholipase A2  
papain  
plasmin  
acidic alpha1 glycoprotein  
alpha 1 lipoprotein  
alpha trypsin inhibitor  
beta1 lipoprotein  
hemopexin  
alpha 1 antitypsin  
transferrin  
plasminogen  
platelet derived growth factor (alpha and beta)  
acidic fibroblast growth factor  
basic fibroblast growth factor  
somatotropin release inhibiting hormone  
somatotropin releasing hormone  
superoxide dismutase  
thymosin  
thyrotropin  
thyrotropin releasing hormone  
alpha fetoprotein  
tumor necrosis factor-alpha  
tumor necrosis factor-beta  
vasoactive intestinal opeptide  
von Willebrand factor  
tissue plasminogen activator  
gondatropin releasing hormone  
parathyroid hormone

antithrombin III  
 protein C  
 protein S  
 activated protein C  
 interferon alpha  
 interferon beta  
 interferon gamma  
 ferritin  
 haptoglobin  
 HDL  
 LDL  
 VLDL  
 TGF (alpha and beta)  
 Steel Factor (stem cell growth factor)  
 HB-epidermal growth factor  
 FGF-6  
 SLF  
 KGF  
 DDNF  
 NT-3

sis oncogene protein  
int-2 oncogene protein  
hst oncogene protein

#### B. Cell Surface Proteins

CD1a	thymocyte cell surface protein
CD1b	cortical thymocyte, dermal cell surface protein
CD1c	cortical thymocyte cell surface protein
CD2	E rosette receptor
CD3	T cell receptor complex
CD4	T helper/inducer cell surface protein
CD5	T cells, B cell, cell surface protein
CD6	Pan T, B cells of CLL cell surface protein
CD7	T cells, NK cell surface protein
CD8	T cytotoxic/suppressor, NK cell surface protein
CD9	Monocytes, Pre-B, platelet cell surface protein
CD10	CALLA, Pre-B, granulocyte cell surface protein
CD11a	LFA-1 Alpha chain
CD11b	Mac 1 (adhesion molecule)
CD11c	p150-95 (adhesion molecule)
CDw12	Monocyte, granulocyte, platelet cell surface protein
CD13	Pan myeloid (CA ++ mobilization) cell surface protein
CD14	Monocyte cell surface protein
CD15	Hapten X (fucosyl N acetylactosamine), granulocyte
CD16	IgG Fc Receptor III, low affinity
CDw17	Lactoceramide
CD18	B chain of LFA-1, Mac 1, p150-95
CD19	Pan B, cell surface protein
CD20	B cells, dendritic reticular cell surface protein
CD21	B cells, dendritic cells, CR2 (EBV Rc) Epstein Barr Virus Receptor

CD22	B cell, cell surface protein
CD23	IgE Fc Receptor low affinity
CD24	B cell, cell surface protein
CD25	IL2 Receptor
CD26	Dipeptylpeptidase IV of activated T lymphocytes
CD27	Mature T cell surface protein
CD28	Tp44 Ag, T cells, plasma cell surface protein
CD29	VLA Beta chain
CD30	Activation antigen
CD31	Myeloid Ag. gp11a Antigen
CD32	IgG Fc Receptor
CD33	Pan myeloid cell surface protein
CD34	Lymphoid and myeloid precursor cell surface protein
CD35	CR1, granulocytes, monocytes, dendritic cell surface protein
CD36	gpIV, thrombospondin receptor
CD37	B cell, cell surface protein
CD38	B & T cells and plasmacyte cell surface protein
CD39	B cells, macrophages, endothelial cell surface protein
CD40	B cells, B lymphocytes carcinoma (BLCa) cell surface protein
CD41a	gpIIb/IIIa
CD41b	gpIIb
CD42a	gpIX
CD42b	gpIb
CD43	T cells, granulocytes, RBC, cell surface protein
CD44	T cells, pre-B, granulocytes, cell surface protein
CD45	Leukocyte common antigen (LCA)
CD45Ra	Restricted LCA, subset of CD4 + T cells
CD45Rb	Leukocyte cell surface protein
CD45Ro	Restricted LCA
CD46	Membrane Cofactor Protein (MCP)
CD47	N-linked glycan
CD48	Leukocytes (PI-PLC linked)
CDw49a	a1 VLA chain
CDw49b	gp1aIIa, a2 VLA chain, collagen receptor
CDw49c	a3 VLA chain
CDw49d	a4 VLA chain
CDw49e	gp1c, a5 VLA chain
CDw49f	gp1cIIa, a6 VLA chain, laminin receptor
CDw50	Leukocyte cell surface protein
CD51	a chain vitronectin Rc (VNR) receptor
CDw52	Campath-1, leukocyte cell surface protein
CD53	Leukocyte cell surface protein
CD54	ICAM-1 (Intracellular Adhesion Molecule), leukocytes
CD55	DAF (Decay Accelerating Factor)
CD56	N-CAM (NKH-1), Adhesion Molecule
CD57	HNK1, Natural Killer cell surface protein
CD58	Leukocyte functional antigen cell surface protein
CD59	Leukocyte cell surface protein
CDw60	Neu AC-Neu Gal, T lymphocytes subset
CD61	gpIIIa, VNR B chain, Integrin B3
CD62	GMP-140 (PADGEM)
CD63	Activated platelet cell surface protein

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CD64	Fc receptor, monocytes
CDw65	Fucoganglioside
CD66	Granulocyte cell surface protein
CD67	Granulocyte (PI linked) cell surface protein
CD68	Macrophage cell surface protein
CD69	Activation Inducer Molecule
CDw70	Activated B & T cells, Reed Sternberg cell, cell surface protein
CD71	Transferrin receptor
CD72	Pan B cell surface protein
CD73	Ecto5'Nucleotidase
CD74	Class II associated invariant chain
CDw75	Mature B cell surface protein
CD76	Mature B cells, T cell subset, granulocyte cell surface protein
CD77	Globotriaosylceramide (Gb3), Burkitt's lymphoma cell surface protein
CDw78	Pan B (monocyte) cell surface protein
ICAM-1	Thrombin Receptor
ICAM-2	p-glycoprotein (MDR-1 gene product)
LPAM-2	(MDR-2 gene product)
VCAM-1	
ELAM-1	
T-cell receptor	
LAM-1	

#### Histocompatibility antigens (Cell surface antigens)

HLA-A1, HLA-A2, HLA-A3, HLA-A11, HLA-A23(9), HLA-A24(9), HLA-A25(10), HLA-A26(10), HLA-A29(w19), HLA-A30(w19), HLA-A31(w19), HLA-A32(w19), HLA-A33(w19), HLA-Aw34(10), HLA-Aw36, HLA-Aw43, HLA-Aw66(10), HLA-Aw68(28), HLA-Aw69(28), HLA-Aw74(w19), HLA-Bw4(4a), HLA-Bw6(4b), HLA-B7, HLA-B8, HLA-B13, HLA-B18, HLA-B27, HLA-B35, HLA-B37, HLA-B38(16), HLA-B39(16), HLA-Bw41, HLA-Bw42, HLA-B44(12), HLA-B45(12), HLA-Bw46, HLA-Bw47, HLA-Bw48, HLA-B49(21), HLA-Bw50(21), HLA-B51(5), HLA-Bw52(5), HLA-Bw53, HLA-Bw54(22), HLA-Bw55(22), HLA-Bw56(22), HLA-Bw57(17), HLA-Bw58(17), HLA-Bw59, HLA-Bw60(40), HLA-Bw61(40), HLA-Bw 62(15), HLA-Bw63(15), HLA-Bw64(14), HLA-Bw65(14), HLA-Bw67, HLA-Bw71(70), HLA-Bw72(70), HLA-Bw73, HLA-Bw75(15), HLA-Bw76(15), HLA-Bw77(15), HLA-Cw1, HLA-Cw2, HLA-Cw3, HLA-Cw4, HLA-Cw5, HLA-Cw6, HLA-Cw7, HLA-Cw8, HLA-Cw9(3), HLA-Cw10(3), HLA-Cw11, HLA-Dw1, HLA-Dw2, HLA-Dw3, HLA-Dw4, HLA-Dw5, HLA-Dw8, HLA-Dw9, HLA-Dw10, HLA-Dw11(7), HLA-Dw12, HLA-Dw13, HLA-Dw14, HLA-Dw15, HLA-Dw16, HLA-Dw17(7), HLA-Dw18(w6), HLA-Dw19(w6), HLA-Dw20, HLA-Dw21, HLA-Dw22, HLA-Dw23, HLA-Dw24, HLA-Dw25, HLA-Dw26, HLA-DR1, HLA-DR2, HLA-DR3, HLA-DR4, HLA-DR5, HLA-DRw6, HLA-DR7, HLA-DRw8, HLA-DR9, HLA-DRw10, HLA-DRw11(5), HLA-DRw12(5), HLA-DRw13(6), HLA-DRw14(6), HLA-DRw15(2), HLA-DRw16(2), HLA-DRw17(3), HLA-DRw18(3), HLA-DRw52, HLA-DRw53, HLA-DQw1, HLA-DQw2, HLA-DQw3, HLA-DQw4, HLA-DQw5(w1), HLA-DQw6(w1), HLA-DQw7(w3), HLA-DQw8(w3), HLA-DQw9(w3), HLA-DPw1, HLA-DPw2, HLA-DPw3, HLA-DPw4, HLA-DPw5, HLA-DPw6



Insulin receptor  
Insulin-like growth factor receptor  
Sodium/potassium ATPase  
Sodium/chloride cotransporter  
IL-1 receptor  
IL-3 receptor  
IL-4 receptor  
Parathyroid hormone receptor  
GnRH receptor  
CSF-M receptor  
CSF-GM receptor  
CSF-G receptor  
Erythropoietin receptor  
Complement receptor  
C1b receptor  
EGF receptor  
Follicle stimulating hormone receptor  
Follicle stimulating hormone releasing hormone receptor  
Growth hormone receptor  
Glucagon receptor  
Leutinizing hormone receptor  
Leutinizing hormone releasing hormone receptor  
Growth hormone releasing hormone receptor  
Nerve growth factor receptor  
Melanotropin release inhibiting hormone receptor  
Platelet derived growth factor receptor (alpha and beta)  
Fibroblast growth factor receptor (1 and 2)  
Somatotropin release inhibiting hormone receptor  
Somatotropin releasing hormone receptor  
Thyrotropin receptor  
Thyrotropin releasing hormone receptor  
Tumor necrosis factor - alpha receptor  
Tumor necrosis factor - beta receptor  
Complement C3a receptor  
Complement C5a receptor  
Complement C3b receptor  
Complement CR2 receptor  
Complement CR3 receptor  
CSF-1 receptor  
GMCSF receptor  
SLF receptor  
flg oncogene protein  
c-ros oncogene protein  
erb-B2 oncogene protein  
trk-B oncogene protein  
trk oncogene protein  
c-fms oncogene protein  
c-kit oncogene protein  
erb-B oncogene protein  
HER-2/neu oncogene protein  
kit oncogene protein

C. Virus and Bacterial Targets

## HIV-1/HIV-2

reverse transcriptase (including RNase H)  
protease

integrase

gag proteins (including p17, p24, p15)

tat protein

rev protein

nef protein

vif protein

vpr protein

vpu protein

envelope proteins (including gp 120, gp41)

## HTLV-I/II

gag proteins (including gp24, gp19, gp15)

protease

pol (including reverse transcriptase and RNase H)

envelope genes (including gp46 and gp41)

tax

rex

## Human papillomaviruses

E7 protein

E6 protein

E6\* protein

E4 protein

E1 proteins

E1-E4 proteins

E2 proteins

capsid proteins (L1 and L2)

## Influenza A and B

polymerase proteins (including PA, PB1, and PB2)

hemagglutinin (HA)

neuraminidase (NA)

nucleoprotein (NP)

M1 and M2 proteins

NS1 and NS2 proteins

## Hepatitis B

Envelope (surface antigen) proteins (including pre-S1, pre-S2 and S)

Nucleocapsid (core) proteins

P-gene product

X-gene product

**Cytomegalovirus**

Immediate early (alpha) gene products (including IE1 and IE2)  
Early (beta) gene products (including DNA pol p140, DBP52 EDBP 140)  
Late (gamma) structural gene products

**Herpes Simplex Virus**

thymidine kinase  
ribonucleotide reductase  
virus-encoded envelope glycoproteins

**Epstein-Barr Virus**

immediate early gene products (including ZLF1 protein and RLF1 protein)  
early gene products (including SMLF1, MRF1, ALF2, HRF1, ribonucleotide reductase, thymidine kinase [XLF1])  
virus-encoded glycoproteins

lipopolysaccharides (from gram negative or gram positive bacteria)  
botulinum toxin  
diphtheria toxin  
cholera toxin  
endotoxin

**D. Intracellular Targets (proteins/lipids/etc)****Lipids**

fatty acids  
glycerides  
glycerylethers  
phospholipids  
sphingolipids  
steroids  
fat soluble vitamins  
glycolipid  
phospholipids  
lecithins  
phosphatidic acids (cephalins)  
sphingomyelin  
plasmalogens  
phosphatidyl inositol  
phosphatidyl choline  
phosphatidyl serine  
phosphatidyl inositol  
diphosphatidyl glycerol  
oleic  
palmitic  
stearic acids  
linoleic acid  
acylcoenzyme A

phosphoglyceride  
phosphitidate  
retinoic acid  
retinoids  
lipoprotein A  
proteolipid  
sphingolipids  
sphingosine  
ceramides  
cerebrosides  
gangliosides  
sphingomyelins  
terpenes  
sesquiterpenes  
diterpenes  
triterpenes  
tetraterpenes  
steroids  
cholesterol  
cholesterol esters  
cholic acid  
phosphatidylcholine  
estrogen  
testosterone  
androgens  
2-keto-3-deoxyoctanoate

Intracellular proteins

p53  
pRB (retinoblastoma gene product)  
methemoglobin  
hemoglobin A  
hemoglobin A1  
hemoglobin A2  
hemoglobin Barcelona  
hemoglobin Barts  
hemoglobin Beth Isreal  
hemoglobin Bunbury  
hemoglobin Cochin-Port Royal  
hemoglobin Cowntown  
hemoglobin Cranston  
hemoglobin Creteil  
hemoglobin D  
hemoglobin D-Los Angeles  
hemoglobin D-Punjab  
hemoglobin F  
hemoglobin Gower  
hemoglobin Hammersmith  
hemoglobin Hiroshima  
hemoglobin Indianapolis  
hemoglobin Kansas  
hemoglobin Kariya

hemoglobin Kempsey  
hemoglobin Kenya  
hemoglobin Lepore  
hemoglobin M  
hemoglobin M Hyde Park  
hemoglobin M Iwate  
hemoglobin M Saskatoon  
hemoglobin Nancy  
hemoglobin Philly  
hemoglobin Quong Sze  
hemoglobin Ranier  
hemoglobin Raleigh  
hemoglobin S  
hemoglobin Sealy  
hemoglobin Seattle  
hemoglobin St. Louis  
hemoglobin St. Mande  
hemoglobin Titusville  
hemoglobin Torino  
hemoglobin Wayne  
hemoglobin York  
hemoglobin Zurich

src oncogene protein  
abl oncogene protein  
met oncogene protein  
Ha-ras oncogene protein  
Ki-ras oncogene protein  
N-ras oncogene protein  
fps oncogene protein  
mos oncogene protein  
raf oncogene protein  
pim oncogene protein  
crk oncogene protein  
dbl oncogene protein  
rel oncogene protein  
yes oncogene protein  
fgr oncogene protein  
L-myc oncogene protein  
int-1 oncogene protein  
ets oncogene protein  
bcl-2 oncogene protein

1-acylglycerol-3-phosphate acyltransferase  
3-b-hydroxy-steroid dehydrogenase(EC5.3.3.1)  
3-hydroxybutyrate dehydrogenase  
3-ketothiolase  
5'-nucleotidase  
8-oxoguanosine deglycosylase  
11b-hydroxylase (EC 1.14.15.4)  
18-hydroxylase  
21-steroid hydroxylase(EC 1.14.99.10)  
2,3-oxidosqualene lanosterol cyclase  
24,28-sterol reductase  
a-actin  
a-mannosidase  
a-melogenin  
a-tubulin  
acetolactate synthase  
acetyl glucosaminyl transferase  
acetyl spermine deactylase  
acetyl transacylase  
acetyl-CoA carboxylase  
acetyl-CoA malate citrate synthase      acetyl cholinesterase  
acid phosphatase  
acid protease  
aconitase  
actin  
adenosine deaminase  
adenosylhomocysteine hydrolase  
adenosylmethionine decarboxylase  
adenylate cyclase  
adenylate deaminase  
adenylate kinase  
adenylsuccinate lyase  
adenylsuccinate synthase  
alanine aminotransferase  
alcohol dehydrogenase  
aldolase  
aldose reductase  
alkaline phosphatase  
amidophosphoribosylamine transferase  
AMP phosphodiesterase  
amyloid b/A4 protein  
amyloid precursor protein  
ankarin  
arginase  
argininosuccinate lyase  
argininosuccinate synthetase

aromatase  
aryl sulfatase  
aspartate aminotransferase  
aspartate transcarbamoylase  
ATP diphosphohydrolase  
ATPase  
b-actin  
b-glucuronidase  
b-glycerophosphatase  
b-ketoacyl-ACP reductase  
b-ketoacyl-ACP synthetase  
b-spectrin  
b-tropomyosin  
b-tubulin  
C5a inactivation factor  
calcitonin  
calmodulin  
calpain I  
calreticulin  
carbamoyl-phosphate synthetase  
carbonic anhydrase  
casein kinase 1  
casein kinase 2  
catalase  
catechol methyltransferase  
cathepsin  
cathepsin B and L  
cdc 2 p34  
cdc 10  
cdc 13 p60  
cdc 25 p80  
chaparonin  
cholesterol esterase  
cholesterol monooxygenase  
citrate synthetase  
clathrin  
  
collagenase  
connective tissue activating peptide  
core protein  
cortisol dehydrogenase  
cyclin A and B  
cyclophilin  
cytidine deaminase  
cytidylate deaminase  
cytochrome C peroxidase

cytochrome P450  
cytosine methyl transferase  
defensin  
diacylglycerol acyltransferase  
dihydrofolate reductase  
dihydrouracil dehydrogenase  
dihydroorotase  
dihydroorotate dehydrogenase  
dioxygenase  
dopamine monooxygenase  
dynenin  
elastase  
elastin  
elongation factor Tu  
endo-rhamosidase  
enolase  
enoyl-ACP hydratase  
enoyl-ACP reductase  
fatty acid synthetase  
ferritin  
ferrodoxin  
fructose biphosphate aldolase  
fumarase  
GABA aminotransferase  
galactosidase  
gelatinase  
gelsolin  
glucophosphate isomerase  
glucosylceramide galactosyl transferase  
glutaminase  
glutamine phosphoribosylpyrophosphate amidotransferase  
glycerol phosphate acyl transferase  
glycerol phosphate dehydrogenase  
glycinamide ribonucleotide transfomylase  
GTP binding protein  
heavy meromyosin  
hexokinase  
histaminase  
histidine decarboxylase  
HSP 27  
hydropyrimidine hydrolase  
hydroxy acyl CoA dehydrogenase  
hydroxy steroid dehydrogenase  
hydroxy-methylglutaryl CoA cleavage enzyme  
hydroxy-methylglutaryl CoA reductase  
hydroxy-methylglutaryl CoA sythetase



hypoxanthine-guanine phosphoribosyl transferase  
IMP dehydrogenase  
indole lyase  
inositol phosphate phosphatase  
isocitrate lyase  
kinin generating enzyme  
lactate dehydrogenase  
lactoferrin  
laminin  
leukocyte elastase  
lipocortin  
lipoxygenase  
long chain fatty acid CoA ligase  
lysozyme  
major basic protein  
malate dehydrogenase  
malate synthase  
malonyl transacylase  
mannosidase  
methionine adenosyltransferase  
mixed function oxygenase  
myloperoxidase  
myofilament  
myristoyltransferase  
N-acetyl glucuronidase  
Na/K ATPase  
NAD-dependent sterol 4-carboxylase  
NADase  
NADPH-dependent 3-oxosteroid reductase  
nexin  
nucleolar protein B23  
nucleoside diphosphate kinase  
ornithine aminotransferase  
ornithine carbamoyltransferase  
ornithine decarboxylase  
orotate decarboxylase  
orotate phosphoribosyl transferase  
  
peptidyl prolyl isomerase  
peptidylamidoglycolate lyase  
phenylalanine hydroxylase  
phosphatidate phosphatase  
phosphoenol pyruvate carboxykinase  
phosphofructokinase  
phosphoglucokinase  
phosphoglucomutase

phosphoglycerate kinase  
phosphoglyceromutase  
phospholipase A2  
phospholipase C  
phospholipase CG1  
phospholipase D  
phospholipase S  
phosphoribomutase  
phosphoribosylphosphate transferase  
plasminogen activator inhibitor  
platelet factor 4  
porin  
pRb retinoblastoma gene product  
properidin  
prostaglandin synthase  
Protein kinase C  
purine nucleoside phosphorylase  
pyruvate dehydrogenase  
pyruvate kinase  
ribonucleotide reductase  
ribosephosphate pyrophosphate kinase  
ricin tropoelastin  
serine/threonine kinase  
spectrin  
spermine synthase  
squalene epoxidase  
squalene monooxygenase  
sterol methyltransferase  
suc 1 p13  
succinyl CoA synthetase  
superoxide dismutase  
tartrate dehydrogenase  
thioesterase  
thioredoxin  
thrombospondin  
thromboxane A2 synthetase  
thymidylate synthetase  
transacylase  
triosephosphate dehydrogenase  
triosephosphate isomerase  
tRNA synthetase  
tropomyosin  
tryptophan synthase  
tubulin  
tyrosine kinase  
ubiquinone reductase

uridine monophosphate kinase  
urokinase type plasminogen activator  
vitamin K reductase  
*wee* -1 gene product  
xanthine dehydrogenase  
xanthine oxidase  
xylosyl transferase

#### E. Small Molecules and Other Compounds

2-phosphoglycerate  
3-hydroxy acyl-CoA  
3-phospho-5-pyrophosphomevalonate  
3-phosphoglycerate  
3-phosphohydroxypyruvate  
3-phosphoserine  
5-alpha-dihydrotestosterone  
5-phospho-beta-ribosylamine  
5-phosphoribosyl 1-pyrophosphate  
5-phospho-alpha-ribosyl-1-pyrophosphate  
5'-phosphoribosyl-4-carboxamide-5-aminoimidazole  
6-benzylaminopurine  
17-hydroxyprogesterone  
acetaminophen  
acetyl-coenzyme A  
acetylcholine  
acetylsalicylic acid  
adenine  
adenosine  
ADP  
aflatoxin B1  
aflatoxin G1  
aflatoxin M1  
aldosterone  
allantoin  
allodeoxycholic acid  
allopurinol  
alpha ketoglutarate  
alpha,beta-dihydroxy-beta-methylvalerate  
alpha-aceto-alpha-hydroxybutyrate  
alpha-amino-beta-ketoadipate  
alpha-bungarotoxin  
alpha-carotene  
alpha-keto-beta-methylvalerate  
alpha-keto-glutarate  
alpha-ketobutyrate

alpha-ketoglutarate  
amiloride  
aminopterin  
AMP  
amylopectin  
amylose  
anti-diuretic hormone  
antipyrine  
arachidic acid  
arachidonic acid  
arecoline  
arginine  
argininosuccinate  
ascorbic acid  
aspartate semialdehyde  
aspartyl phosphate  
ATP  
atropine  
bacitracine  
benztropine  
beta-caratene  
betamethazone  
bilirubin  
biliverdin  
biotin  
carbachol  
carbamoyl phosphate  
carboline  
carnitine  
CDP  
cholesterol  
cholic acid  
chorismic acid  
cis aconitate  
citrate  
citrulline  
CMP  
cocaine  
codeine  
Coenzyme Q  
coenzyme A  
corticosterone  
cortisol  
cortisone  
coumarin  
creatine

creatinine  
CTP  
cyanocobalamin  
cyclic AMP  
cyclic CMP  
cyclic GMP  
cyclic TMP  
cystathionine  
cytidine  
cytochrome  
D-Erythrose  
D-Fructose  
D-Galactosamine  
D-glucose  
D-Glucuronic acid  
dADP  
dAMP  
dATP  
dCDP  
dCMP  
dCTP  
delta-4-androstenedione  
deoxyadenosyl cobalamin  
deoxycholic acid  
dGDP  
dGMP  
dGTP  
dihydroorotate  
dihydroxyphenylalanine  
diphosphoglycerate  
dopamine  
dTDP  
dTMP  
dTTP  
dUDP  
dUMP  
dUTP  
eosinophil chemotactic factor of anaaphyaxis-A  
epinephrine  
estriol  
estrone  
ethynylestradiol  
FAD  
farnesyl pyrophosphate  
fatty Acyl-s-CoA  
ferrodoxin

FMN  
FMNH<sub>2</sub>  
folic acid  
fructose 2,6-diphosphate  
fructose  
fructose 1,6-diphosphate  
fructose 6-phosphate  
Fructose1,6-diphosphate  
fumarate  
galactose  
galactose  
GalNAc  
gama-aminolevulinate  
gamma-carotene  
gastric inhibitory protein  
gaunidinoacetate  
GDP  
gentamycin  
glucosamine  
glucosamine 6-phosphate  
glucose  
glucose 1,6-diphosphate  
glucose 1-phosphate  
glucose 6-phosphate  
Glutamate  
glutamate semialdehyde  
glutaryl-CoA  
glutathione  
glyceraldehyde 3-phosphate  
glycerol 1-phosphate  
glychocholate  
glycine  
glyoxylate  
GMP  
GTP  
guanine  
hemicholine  
histamine  
homogentisate  
homoserine  
hydrocortisone  
hydroxyproline  
indole  
inosine  
inositol  
inositol phosphate

intermediate molecular weight eosinophil chemotactic factor of  
isocitrate  
isopentenyl pyrophosphate  
L-alanine  
L-arginine  
L-asparagine  
L-aspartic acid  
L-aspartic acid  
L-azoserine  
L-cysteine  
L-Fucose  
L-glutamic acid  
L-glutamine  
L-histidine  
L-isoleucine  
L-leucine  
L-lysine  
L-malate  
L-methionine  
L-phenylalanine  
L-proline  
L-serine  
L-threonine  
L-tryptophane  
L-tyrosine  
L-valine  
lanosterol  
leukotriene B<sub>4</sub>  
leukotriene C<sub>4</sub>  
leukotriene D<sub>4</sub>  
leukotrienes  
lipoic acid  
luciferin  
malonate  
malonyl-CoA  
methocholine  
methotrexate  
methylenetetrahydrofolate  
methylmalonyl-CoA  
mevalonate  
mevalonate-5-phosphate  
muscarin  
N-Formylmethionine  
NAD  
NADH  
NADP

NADPH  
neostigmine  
nicotinamide  
nicotine  
nicotinic acid  
norepinephrine  
ornithine  
oxaloacetate  
oxotremorine  
p-benzoquinone  
pancuronium  
pantothenic acid  
para-aminobenzoic acid  
phosphenolpyruvate  
phosphocreatinine  
physostigmine  
pilocarpine  
piperidine  
pirenzepine  
plastoquinone  
platelet-activating factor  
porphobilinogen  
pregnenolone  
progesterone  
prolinamide  
propionyl-CoA  
prostaglandin D2  
protoporphyrin IX  
pteridine  
pyridoxal  
pyridoxal phosphate  
pyridoxal phosphate  
pyridoxamine  
pyridoxamine phosphate  
pyrodoxine  
pyroglutamic acid  
pyrophosphate  
pyrrolidine  
pyrroline-5-carboxylate  
pyrrolizidine  
quinplizidine  
quinuclidinylbromide  
RGD peptide  
riboflavin  
ribose  
s-adenosylhomocysteine



s-adenosylmethionine  
scopolamine  
serotonin  
slow-reacting substance of anaphylaxis  
squalene  
suberyldicholine  
succinate  
succinyl-CoA  
taurocholate  
testosterone  
tetrahydrofolic acid  
thiamine  
thioredoxin  
thromboxane A2  
thromboxane B2  
thymine  
tropane  
ubiquinol  
ubiquinone  
UDP  
UDP-galactose  
UMP  
uracil  
urea  
uric acid  
UTP  
vitamin A  
vitamin D  
vitamin E  
vitamin K

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CLAIMS

We claim:

1. A single-stranded DNA aptamer containing at  
5 least one binding region capable of binding specifically  
to a target molecule.
2. An aptamer containing at least one binding  
region capable of binding specifically to a target  
10 molecule that does not normally bind oligonucleotides  
with a dissociation constant ( $K_d$ ) of less than  $20 \times 10^{-9}$ .
3. An aptamer containing at least one binding  
region capable of binding specifically to a target  
15 molecule, wherein the  $K_d$  with respect to the aptamer and  
said target molecule is less by a factor of at least 5,  
as compared to the  $K_d$  for said aptamer and other  
unrelated molecules.
- 20 4. An aptamer containing at least one binding  
region capable of binding specifically to a target  
molecule wherein said binding region contains less than  
15 nucleotide residues.
- 25 5. An aptamer containing at least one binding  
region capable of binding specifically to a target  
molecule wherein said aptamer contains less than 16  
nucleotide residues.
- 30 6. An aptamer containing at least one binding  
region capable of binding specifically to a target  
molecule selected from the group consisting of:  
bradykinin, PGF2 $\alpha$ , CD4, HER2, IL-1 receptor,  
Factor X, and thrombin.

35

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7. The aptamer of claims 1-5 wherein the target molecule exhibits one or more biological functions.

5                   8. The aptamer of claim 7 wherein the target molecule does not exhibit the biological function of binding nucleic acids.

9. The aptamer of claim 1-5 wherein the target  
10 molecule is a protein or peptide.

10. The aptamer of claim 9 wherein the target molecule is an extracellular protein.

15                   11. The aptamer of claim 10 wherein the extracellular protein is selected from the group consisting of botulinum toxin and diphtheria toxin, collagenase, tumor necrosis factor, antithrombin III, interleukins, elastase, and PDGF ( $\alpha$  and  $\beta$ ) fibroblast  
20 growth factors.

12. The aptamer of claim 9 wherein the target molecule is an intracellular protein.

25                   13. The aptamer of claim 12 wherein the intracellular protein is selected from the group consisting of oncogene proteins, hydroxymethyl glutaryl CoA synthase and dihydrofolate reductase.

30                   14. The aptamer of claim 9 wherein the target molecule is a cell surface protein.

35                   15. The aptamer of claim 14 wherein the cell surface protein is selected from the group consisting of

HLA antigens, tumor necrosis factor receptors, EGF receptor, CD62, ICAM-1, ICAM-2, VCAM-1 and ELAM-1.

16. The aptamer of claim 9 wherein the target  
5 molecule is a glycoprotein.

17. The aptamer of claims 1-5 wherein the target molecule is a carbohydrate.

10 18. The aptamer of claim 17 wherein the carbohydrate is a selected from the group consisting of monosaccharide, disaccharide, polysaccharide, or is a glucosaminoglycan or fragment thereof.

15 19. The aptamer of claims 1-5 wherein the target molecule is a lipid.

20 20. The aptamer of claim 19 wherein the lipid is a glycolipid.

21. The aptamer of claim 19 wherein the lipid is a steroid, or triglyceride.

25 22. The aptamer of claims 1-5 wherein the target molecule is a small molecule selected from the group consisting of aflatoxin, histamine, and eicosanoids.

30 23. The aptamer of claims 1-5 wherein the target molecule has a molecular weight from about 100 to about 1000 daltons.

35 24. The aptamer of claims 1-5 wherein the target molecule has a molecular weight from about  $10^3$  to about  $10^4$  daltons.

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25. The aptamer of claims 1-5 wherein the target molecule has a molecular weight from about  $10^4$  to about  $10^6$  daltons.

5

26. The aptamer of claims 1-6 which contains a binding region of less than 14 nucleotide residues.

27. The aptamer of claims 1-6 which contains a binding region of less than 10 nucleotide residues.

10

28. The aptamer of claims 1-4 or 6 which contains 6-100 nucleotide residues.

15

29. The aptamer of claims 1-4 or 6 which contains 6-50 nucleotide residues.

20

30. The aptamer of claims 1-29 wherein said aptamer is capable of binding specifically to a target molecule at physiological conditions.

25

31. The single-stranded DNA aptamer of claims 1-30 wherein said aptamer binds to said target with a  $K_d$  of less than  $20 \times 10^{-9}$ .

32. The aptamer of claim 31 wherein said aptamer binds to the target with a  $K_d$  of less than  $20 \times 10^{-9}$  at physiological conditions.

30

33. The aptamer of claims 1-32 wherein the  $K_d$  with respect to the aptamer and said target molecule is less by a factor of at least 5, as compared to the  $K_d$  for said aptamer and other unrelated molecules.

35

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34. The aptamer of claims 1-33 wherein the aptamer contains at least one modified linking group, sugar residue and/or base.

5                   35. The aptamer of claim 34 wherein  
the aptamer contains at least one linking group  
wherein P(O)O is replaced by P(O)S, P(S)S, P(O)NR<sub>2</sub>,  
P(O)R, P(O)OR', CO or CH<sub>2</sub>, wherein each R or R' is  
independently H or substituted or unsubstituted alkyl  
10 (1-20C) optionally containing an ether (-O-) linkage,  
aryl, alkenyl, cycloalkyl, cycloalkenyl or aralkyl; or  
the aptamer contains at least one linking group  
attached to an adjacent nucleotide through S or N; or  
the aptamer contains at least one modified form  
15 of purine or pyrimidine or at least one abasic site; or  
the aptamer contains at least one modified or  
analogous sugar other than underivatized ribose.

36. The aptamer of claim 35 which contains at  
20 least one linking group wherein P(O)O is replaced by  
P(O)S and said linking group is attached to each adjacent  
nucleotide through O; or which contains at least one  
linking group wherein P(O)O is replaced by  
P(O)NH(CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>) and said linking group is attached to  
25 each adjacent nucleotide through O; or contains at least  
one uracil (dU) base substituted for thymine; or contains  
at least one abasic site; or contains at least one 5-  
pentynyluracil base substituted for thymine, or contains  
a 2'-O-alkyl, 2'-O-allyl, 2'-S-alkyl, 2'-S-allyl or halo  
30 sugar residue.

37. The aptamer of claims 1-36 which is a  
secondary aptamer.

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38. A method for obtaining an aptamer containing at least one binding region that specifically binds a target, which method comprises:

5 (a) incubating said target with a mixture of member oligonucleotides under conditions wherein the target complexes with some, but not all, members of the mixture to form oligonucleotide-target complexes;

(b) separating the oligonucleotide-target complexes from uncomplexed oligonucleotides;

10 (c) recovering and amplifying the complexed oligonucleotides from said complexes to obtain an aptamer; and

(d) optionally determining the sequence of the recovered aptamer,

15 wherein said aptamer is a single-stranded DNA, or

wherein said aptamer contains at least one binding region capable of binding specifically to a target molecule that does not normally bind  
20 oligonucleotides with a dissociation constant ( $K_d$ ) of less than  $20 \times 10^{-9}$ , or

wherein said aptamer contains at least one binding region capable of binding specifically to a target molecule, wherein the  $K_d$  with respect to the  
25 aptamer and said target molecule is less by a factor of at least 5, as compared to the  $K_d$  for said aptamer and other molecules, or

wherein said aptamer contains at least one binding region capable of binding specifically to a  
30 target molecule wherein said binding region contains less than 15 nucleotide residues, or

wherein said aptamer contains at least one binding region capable of binding specifically to a

35

target molecule wherein said aptamer contains less than 16 nucleotide residues, or

wherein said aptamer contains at least one binding region capable of binding specifically to a target molecule selected from the group consisting of exemplified targets.

39. The method of claim 38 wherein said mixture of oligonucleotides contains at least one modified oligonucleotide.

40. The method of claim 38 wherein said amplifying is conducted using at least one modified nucleotide.

41. The method of claims 38-40 wherein said mixture of oligonucleotides contains at least one randomized-sequence region.

42. The method of claims 38-41 which further includes repeating steps (a)-(c) using the recovered and amplified complexed oligonucleotides resulting from step (c) in succeeding step (a).

43. The method of claims 38-42 wherein the  $K_d$  with respect to the oligonucleotide mixture and target is at least 50-fold more than the  $K_d$  with respect to the aptamer and target.

44. An aptamer prepared by the method of claims 38-43.

45. A method to obtain a secondary aptamer that specifically binds to a target molecule which method comprises:



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- 5 (a) incubating said target molecule with a mixture of oligonucleotide sequences under conditions wherein complexation occurs with some, but not all, members of the mixture to form oligonucleotide-target complexes;
- (b) separating the oligonucleotide-target complexes from uncomplexed oligonucleotides;
- (c) recovering and amplifying the complexed oligonucleotides from said complexes;
- 10 (d) optionally repeating steps (a)-(c) with the recovered oligonucleotides of step (c);
- (e) determining the sequences of the recovered oligonucleotides;
- (f) determining a consensus sequence included
- 15 in the recovered oligonucleotides; and
- (g) synthesizing a secondary aptamer which comprises the consensus sequence.

20 46. A secondary aptamer prepared by the method of claim 45.

47. A method for obtaining an aptamer containing at least one binding region that specifically binds a target, which method comprises:
- 25 (a) incubating said target with a mixture of member oligonucleotides under conditions wherein the target complexes with some, but not all, members of the mixture to form oligonucleotide-target complexes;
- (b) separating the oligonucleotide-target
- 30 complexes from uncomplexed oligonucleotides;
- (c) recovering and amplifying the complexed oligonucleotides from said complexes to obtain an aptamer; and
- (d) optionally determining the sequence of the
- 35 recovered aptamer,

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wherein the dissociation constant ( $K_d$ ) with respect to said target and mixture of oligonucleotides is  $\geq 1 \mu M$ , or

5 wherein the  $K_d$  with respect to the aptamer and said target is less by a factor of at least 50 as compared to the  $K_d$  for said target and said mixture of oligonucleotides; or

wherein steps (a) and (b) are conducted under physiological conditions, or

10 wherein said mixture of oligonucleotides consists of single-stranded DNA.

48. The method of claim 47 wherein said mixture of oligonucleotides contains at least one  
15 modified oligonucleotide.

49. The method of claim 47 wherein said amplifying is conducted using at least one modified  
nucleotide.

20

50. The method of claims 47-49 wherein said mixture of oligonucleotides contains at least one randomized-sequence region.

25

51. The method of claims 47-49 which further includes repeating steps (a)-(c) using the recovered and amplified complexed oligonucleotides resulting from step (c) in succeeding step (a).

30

52. The method of claim 47 wherein said mixture of oligonucleotides is of undetermined sequence.

53. An aptamer prepared by the method of  
35 claims 47-52.

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54. A method to obtain an aptamer containing a binding region which specifically binds a target molecule which comprises:

- 5 (a) incubating the target molecule reversibly coupled to a support with a mixture of oligonucleotide sequences under conditions wherein the coupled target molecule complexes with some, but not all, members of the mixture to form support-bound oligonucleotide complexes;
- 10 (b) decoupling and recovering the oligonucleotide target complex from the support to obtain free aptamer-target complexes;
- (c) recovering and amplifying the complexed oligonucleotides from the free oligonucleotide-target complexes to obtain a population of aptamers;
- 15 (d) optionally repeating steps (a)-(c) using as said mixture the recovered population of aptamers of step (c); and
- (e) optionally determining the sequence of the recovered aptamers.
- 20

55. The method of claim 54 wherein in step (a) the target substance is reversibly coupled to the support using an activated thiol group on the support.

25

56. The method of claim 54 wherein in step (b), decoupling is accomplished by adding a reducing agent.

30 57. The method of claim 56 wherein the reducing agent is dithiothreitol or  $\beta$ -mercaptoethanol.

58. The method of claim 54 wherein the support is a lectin support and the target substance binds reversibly to lectin.

35

59. The method of claim 58 wherein in step (b), decoupling is accomplished by adding a monosaccharide.

5

60. The method of claim 59 wherein the monosaccharide is selected from the group consisting of  $\alpha$ -methyl-mannoside, N-acetylglucosamine, glucose, N-acetylgalactosamine and galactose.

10

61. A method for obtaining aptamers capable of binding a target, said method comprising:

(a) providing a first pool of oligonucleotides of unpredetermined sequence, said pool comprising a quantity of oligonucleotides sufficiently reflective of the structural complexity of said target as to ensure the presence of at least one oligonucleotide capable of binding said target;

(b) incubating said pool of oligonucleotides, or a portion thereof with said target under conditions wherein complexation occurs between some oligonucleotides and said target, said complexed oligonucleotides defining a first aptamer population;

(c) recovering said first aptamers in substantially single stranded form from uncomplexed oligonucleotides;

(d) attaching a known nucleotide sequence to at least one end of said first aptamers;

(e) amplifying said first aptamers;

(f) removing said known nucleotide sequence from said first aptamers;

30

(g) optionally repeating steps (a)-(f) a sufficient number of times to generate an optimal aptamer population having high affinity for target.

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62. A method for obtaining an oligonucleotide capable of complexing to a desired target, said oligonucleotide being substantially non-predetermined sequence, said method comprising:

- 5 (a) incubating said target with a pool of oligonucleotides of non-predetermined or substantially non-predetermined sequence under conditions wherein some, but not all, oligonucleotides complex with said target;
- (b) separating oligonucleotide:target  
10 complexes;
- (c) recovering the oligonucleotides from step b in substantially single stranded form;
- (d) attaching a first linker to the 5' end of said oligonucleotide and a second linker to the 3' end of  
15 said oligonucleotide, both said 5' and said 3' linkers of known nucleotide sequence, thereby generating an oligonucleotide having a 5' linker portion, an oligonucleotide portion and a 3' linker portion;
- (e) amplifying the oligonucleotide of step d,  
20 thereby generating a duplex comprising a first strand having a 5' linker complement portion, an oligonucleotide complement portion and a 3' linker complement portion, and a second strand comprising a 5' linker portion, an oligonucleotide portion and a 3' linker portion;
- 25 (g) removing said 3' linker portion and said 5' linker portion;
- (h) recovering said oligonucleotide in substantially single stranded form.

30 63. The method of claim 62 wherein said 5' linker has a restriction enzyme recognition site at or near the 3' end thereof and said 3' linker has a restriction enzyme recognition site at or near the 5' end thereof.

35

64. The method of claim 63 wherein said 3' linker portion is removed by attaching said duplex to solid support; digesting said attached duplex with a restriction enzyme capable of recognizing the restriction enzyme site at the 5' end thereof.

65. A method for obtaining an aptamer containing at least one binding region that specifically binds a target which method comprises:

- 10 (a) incubating said target molecule with a mixture of oligonucleotides under conditions wherein complexation occurs with some, but not all, members of the mixture to form oligonucleotide-target complexes;
- (b) separating the oligonucleotide-target
- 15 complexes from uncomplexed oligonucleotide;
- (c) recovering and amplifying the complexed oligonucleotide from said complexes; and
- (d) optionally determining the sequence of the recovered oligonucleotide,
- 20 wherein said amplifying is conducted using at least one modified nucleotide, or
- wherein said mixture of oligonucleotides contains at least one modified oligonucleotide.

25 66. A method to obtain an aptamer which specifically binds a first target and fails to bind a second substance, which method comprises:

- incubating said first target with a mixture of member oligonucleotides under conditions wherein
- 30 complexation occurs with some, but not all, members of said mixture;
- separating complexed from uncomplexed oligonucleotides;

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recovering the complexed oligonucleotides to  
provide a first aptamer population;

incubating said second substance with said  
first aptamer population under conditions wherein  
5 complexation occurs with some, but not all, members of  
said mixture;

separating complexed from uncomplexed  
oligonucleotides;

recovering the uncomplexed oligonucleotides to  
10 provide a second aptamer population which specifically  
binds the first target; and

recovering and amplifying the  
oligonucleotide(s) in said second aptamer population.

15 67. A method to obtain an aptamer which  
specifically binds a first target and does not bind to a  
second substance, which method comprises:

contacting said second substance with a mixture  
of oligonucleotides under conditions wherein some but not  
20 all of the members of the mixture bind to the second  
substance;

separating away those members which do not bind  
to the second substance to obtain a first pool of  
oligonucleotides;

25 contacting the first pool with said first  
target;

separating away and isolating those  
oligonucleotides which bind to the first target to  
provide a second pool of aptamers;

30 recovering and amplifying the aptamers.

68. An aptamer prepared by the method of any  
of claims 47-67.

35

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69. A complex formed by a target molecule and the aptamer of claims 1-37, 44, 46, 53 or 68.

70. A method to detect the presence or absence  
5 of a target molecule, which method comprises contacting a sample suspected of containing said target molecule with the aptamer of claims 1-37, 44, 46, 53 or 68 under conditions wherein a complex between said target molecule and the aptamer is formed, and  
10 detecting the presence or absence of said complex.

71. A method to purify a target molecule, which method comprises contacting a sample containing  
15 said target molecule with the aptamer of claims 1-37, 44, 46, 53 or 68 attached to solid support under conditions wherein said target molecule is bound to the aptamer coupled to solid support;  
20 washing unbound components of the sample; and recovering the target molecule from said solid support.

72. A pharmaceutical composition for medical use comprising the aptamer of claims 1-37, 44, 46, 53 or  
25 68 in admixture with a physiologically acceptable excipient.

73. A composition for diagnostic use which comprises the aptamer of claims 1-37, 44, 46, 53 or 68.  
30

74. The aptamer of claims 1-37, 44, 46, 53 or 68 coupled to an auxiliary substance.

75. The aptamer of claim 74 wherein said  
35 auxiliary substance is selected from the group consisting



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of a drug, a toxin, a solid support, and specific binding reagent, a label, a radioisotope or a contrast agent.

76. A conjugate for modulating immune response  
5 to a pathologic cell, comprising:  
a targeting agent moiety that specifically  
binds to a surface feature of the pathologic cell; and  
an immunomodulatory moiety that induces an  
immunological response different from that elicited by  
10 the pathologic cell itself in the absence of the  
conjugate.

77. A conjugate according to claim 76, wherein  
said targeting agent is selected from the group  
15 consisting of oligonucleotides, antibodies and ligands  
for cell surface receptors.

78. A conjugate according to claim 77, wherein  
said targeting agent is the aptamer of claims 1-37, 44,  
20 46, 53 or 68.

79. A conjugate according to claim 76, wherein  
the immunomodulatory moiety is selected from the group  
consisting of peptides and carbohydrates.  
25

80. A method for preparing a conjugate for  
modulating immune response to a pathologic cell,  
comprising:  
identifying a targeting agent that specifically  
30 binds to a surface antigen of the pathologic cell; and  
associating said targeting agent with an  
immunomodulatory moiety that induces a desired immune  
response.

35

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81. A method for modulating immune response to  
a pathologic cell, comprising:

administering an amount effective to modulate  
immune response of a conjugate in accordance with

5 claim 76.

10

15

20

25

30

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1 / 2

#		*																	*			
1		26	G	G	G	T	T	G	G	-	-	G	T	C	G	G	T	T	G	G	T	
2		20	G	G	G	A	T	G	G	-	-	T	T	T	G	G	T	T	G	G	G	
3		32	A	G	G	T	T	G	G	-	-	-	G	A	G	G	G	T	G	G	G	
4		41	T	G	G	T	T	G	G	-	-	C	G	A	G	G	A	T	G	G	A	
5		56	A	G	G	T	T	G	G	-	G	T	A	G	T	G	T	T	G	G	T	
6		29	A	G	G	T	T	G	G	-	-	G	C	T	G	G	T	T	G	G	G	
7		17	G	G	G	T	T	G	G	-	-	-	G	A	G	G	T	T	G	G	A	
8		44	T	G	G	T	T	G	G	-	-	G	T	C	G	G	T	T	G	G	G	
9		50	G	G	G	A	T	G	G	-	-	T	G	T	G	G	T	T	G	G	C	
10		30	T	G	G	T	T	G	G	-	-	C	A	G	G	G	A	T	G	G	G	
11		25	T	G	G	A	T	G	G	-	-	T	G	A	G	G	T	T	G	G	A	
12		28	G	G	G	G	T	G	G	-	-	T	T	A	G	G	T	T	G	G	T	
13		47	A	G	G	G	T	G	G	-	-	T	T	A	G	G	T	T	G	G	T	
14		42	C	G	G	T	T	G	G	-	G	T	T	G	G	G	A	T	G	G	A	
15		41	C	G	G	T	T	G	G	-	-	T	G	T	G	G	T	T	G	G	T	
16		53	A	G	G	T	T	G	G	-	-	T	G	T	G	G	G	T	G	G	G	
17		15	C	G	G	G	T	G	G	-	-	A	T	A	G	G	T	T	G	G	A	
18		24	G	G	T	G	T	G	G	T	A	G	T	T	T	G	T	T	G	G	G	
19		23	T	G	G	T	T	G	G	T	T	A	C	T	G	G	T	T	G	G	G	
20		27	G	G	G	T	T	G	G	-	-	T	C	T	G	G	G	T	G	G	A	
21		36	T	G	G	T	T	G	G	-	-	G	T	T	G	G	G	T	G	G	A	
22		25	T	G	G	T	T	G	G	-	-	C	C	A	G	G	T	T	G	G	A	
23		12	C	T	A	G	C	G	G	-	C	A	G	T	G	G	T	T	G	G	G	
24		25	T	G	G	G	T	G	G	-	-	G	G	A	G	G	T	T	G	G	T	
25		49	A	G	G	T	T	G	G	-	-	T	T	T	G	G	G	T	G	G	T	
26		28	A	G	G	T	T	G	G	-	T	T	A	G	G	G	T	T	G	G	T	
27		18	G	G	G	A	T	G	C	-	-	G	G	T	G	G	T	T	G	G	G	
28		55	T	G	G	T	T	G	G	-	T	T	A	T	G	G	T	T	G	G	T	
29		23	A	G	G	T	T	G	G	-	-	T	G	T	G	G	T	T	G	G	C	
30		40	A	G	G	T	T	G	G	-	-	T	G	T	G	G	G	T	G	G	G	
31		41	T	G	G	T	T	G	G	-	-	-	G	A	G	G	T	T	G	G	T	
32		42	G	G	G	T	T	G	G	T	G	G	G	T	G	G	A	T	G	G	T	
								Consensus Sequence														
				G	G	T	T	G	G		(N)3				G	G	T	T	G	G		
	G		9	31	30	6	0	32	31						30	32	6	0	32	32	11	
	A		9	0	1	4	0	0	0						0	0	4	0	0	0	8	
	T		10	1	1	22	31	0	0						2	0	22	32	0	0	11	
	C		4	0	0	0	1	0	1						0	0	0	0	0	0	2	

FIG. 1

SUBSTITUTE SHEET

2 / 2

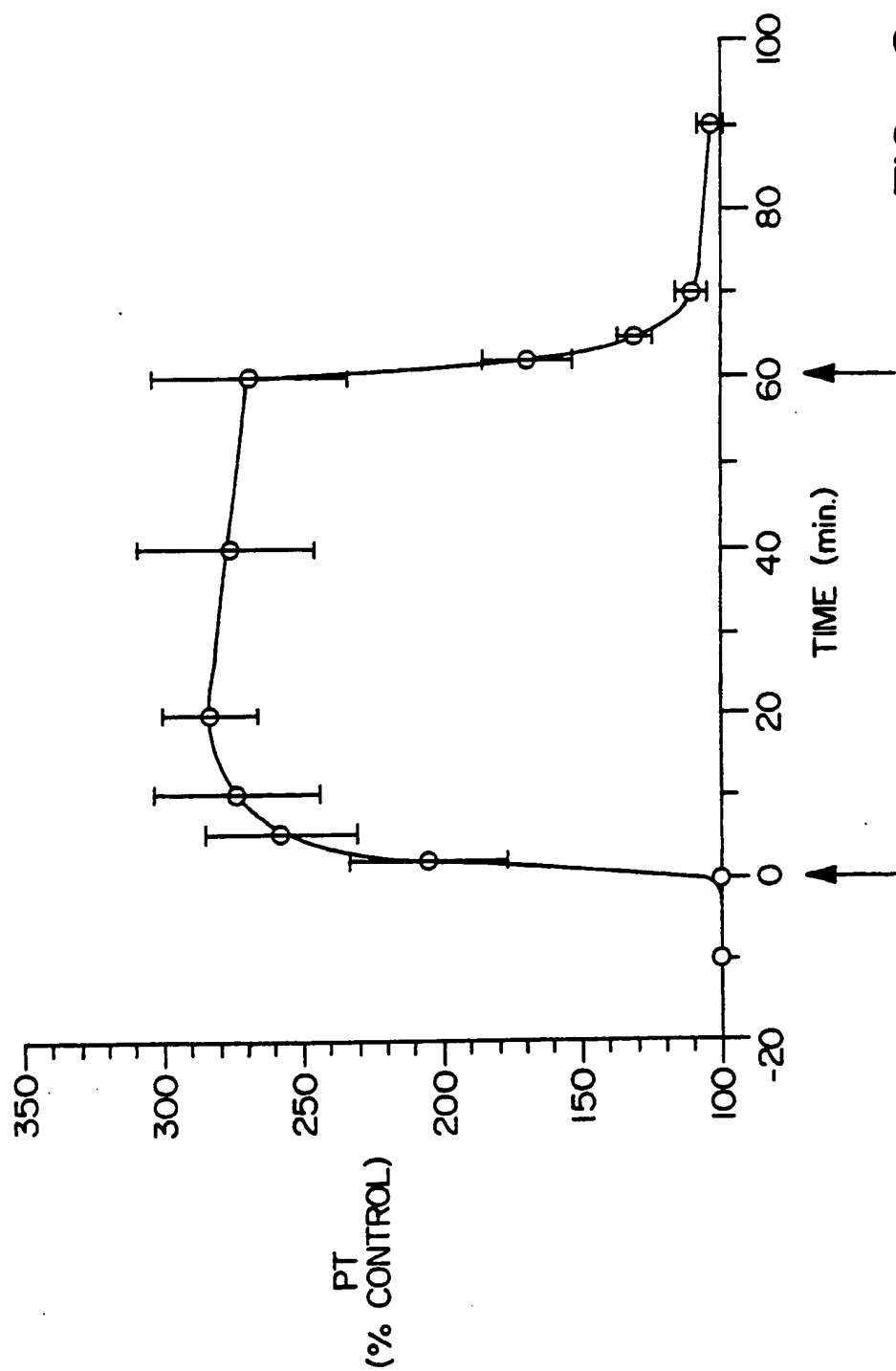
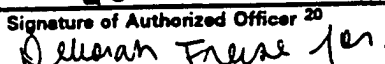


FIG. 2

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/01383

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): C12Q 1/68; C07H 15/12, 17/00 US CL : 435/6, 536/27, 28, 29		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>4</sup>		
Classification System	Classification Symbols	
U.S.	435/6, 536/27, 28, 29	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>5</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category <sup>9</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
y	Nature, Volume 346, Issued 30 August 1990, Ellington et al. "In vitro Selection of RNA Molecules that Bind Specific Ligands," pages 818-822, see entire document.	1-79
y	Proceedings of the National Academy of Sciences, Vol. 32, issued November 1985, Huynh-Dinh et al. "Modified Oligonucleotides as Alternatives to the Synthesis of Mixed Probes for the Screening of cDNA Libraries," pages 7510-7514, see abstract and page 7510, column 1, paragraph 3.	1-79
y	Science, Volume 249, issued 03 August 1990, Tuerk et al., "Systematic Evolution of Ligands by Exponential Enrichment: RNA Ligands to Bacteriophage T4 DNA Polymerase," pages 505-510.	1-79
y	Science, Volume 250, issued 23 November 1990, Blackwell et al., "Differences and Similarities in DNA-Binding Preferences of MyoD and E2A Protein Complexes Revealed by Binding Site Selection," pages 1104-1110	1-79
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>15</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <sup>2</sup>		Date of Mailing of this International Search Report <sup>2</sup>
24 APRIL 1992		15 MAY 1992
International Searching Authority <sup>1</sup>		Signature of Authorized Officer <sup>20</sup>
ISA/US		 L. Yuan

**FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET.		
y	Kirk-Othmer, "Encyclopedia of Chemical Technology, Third Edition, Volume 6" published 1979 by John-Wiley and Sons (NY), see pages 35-54.	71
y	US, A. 4,748,156 (Aoki et al.) 31 May 1988, see entire	6

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE NOT SEARCHED

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

\_\_\_\_\_ cannot be searched by this Authority, namely:

- This international search report has not been established in accordance with the provisions of Article 17, paragraph 1, of the Patent Cooperation Treaty.
1. ☐ Claim numbers \_\_, because they relate to subject matter (1) not required to be searched by this Authority, namely:
2. ☐ Claim numbers \_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:
3. ☐ Claim numbers \_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

VI. ☐ OBSERVATIONS WERE OBTAINED

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

**Remark on protest**

- ☐ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
y	Nucleic Acids Research, Vol 17, No. 10, issued 1989, Kinzler et al, " Whole Genome PCR: Application to the Identification of Sequences Bound by Gene Regulatory Proteins," pages 3645-3653, see entire document.	1-79